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(54) Title: HUMANIZED AND CHIMERIC MONOCLONAL ANTIBODIES

(57) Abstract

The invention relates to new humanized monoclonal antibody comprising an artificial modified consensus sequence at least of the FRs of the heavy chain variable region of a human immunoglobulin. The invention relates, furthermore, to corresponding humanized and chimeric monoclonal antibodies which are binding to epitopes of the Epidermal Growth Factor wherein the responsible hypervariable regions have the following amino acid sequence: *light chain*:CDR-1: -Ser-Ala-Ser-Ser-Ser-Val-Thr-Tyr-Met-Tyr-; CDR-2: -Asp-Thr-Ser-Asn-Leu-Ala-Ser-; CDR-3: -Gln-Gln-Trp-Ser-Ser-His-Ile-Phe-Thr-; *heavy chain*: CDR-1: -Ser-His-Trp-Met-His-; CDR-2: -Glu-Phe-Asn-Pro-Ser-Asn-Gly-Arg-Thr-Asn-Glu-Lys-Phe-Lys-Ser-; CDR-3: -Arg-Asp-Tyr-Asp-Tyr-Asp-Gly-Arg-Tyr-Phe-Asp-Tyr-. The antibodies can be used for therapeutical and diagnostic purposes.

Humanized and Chimeric Monoclonal Antibodies

5

TECHNICAL FIELD OF THE INVENTION

10   The invention relates to new humanized monoclonal antibodies
comprising an artificial modified consensus sequence at least
of the FRs in the variable region of the heavy chain of human
immunoglobulins.

15   The invention relates, furthermore, to humanized and chimeric
monoclonal antibodies which are binding to epitopes of the
Epidermal Growth Factor. The invention discloses the amino
acid sequences of the responding antigen-binding site for
this receptor.

20
The invention relates to pharmaceutical compositions compris-
ing the said antibodies for the purposes of treating tumors
like melanoma, glioma or carcinoma. The said antibodies can
be used also for diagnostic applications regarding locating
25   and assessing the said tumors in vitro or in vivo.

The specification relates to several technical terms which
are here defined as follows:

30

"Humanized" antibodies mean antibodies comprising FRs of the variable regions and constant regions of amino acids located in the light and heavy chain which derive from human sources whereas the hypervariable regions derive from non-human sources.

"Chimeric" antibodies mean antibodies comprising variable and hypervariable regions which derive from non-human sources whereas the constant regions derive from human origin.

"FRs" mean the framework regions of an antibody and are found within the variable regions. In these regions a certain alteration of amino acids occurs.

"CDRs" mean the complementarity determining or "hypervari-able" regions of an antibody and are found within the variable regions. These regions represent the specific antigen-binding site and show an immense exchange of amino acids. CDRs are primarily responsible for the binding affinity of the antigen.

"Consensus sequence" means a non-naturally occurring amino acid sequence as light or heavy chain variable regions and is used as substitute for the originally present non-human heavy or light chain variable regions. The consensus sequences is synthetic and therefore an artificial sequence of the most common amino acids of a distinct class or subclass or sub-group of heavy or light chains of human immunoglobluins.

"EGF" and "EGFR" mean the Epidermal Growth Factor and its receptor.

"V_L" regions mean light chain variable regions.

"V_H" regions mean heavy chain variable regions.

5   BACKGROUND OF THE INVENTION

The murine monoclonal antibody 425 (MAb 425) was raised against the human A431 carcinoma cell line and found to bind to a polypeptide epitope on the external domain of the human
10   epidermal growth factor receptor (EGFR). It was found to inhibit the binding of epidermal growth factor (EGF) at both low and high affinity EGFR sites (Murthy et al., 1987), Enhanced expression of EGFR is found to occur on malignant tissue from a variety of sources thus making MAb 425 a possi-
15   ble agent for the diagnosis and therapeutic treatment of human tumors. Indeed, MAb 425 was found to mediate tumor cytotoxicity in vitro and to suppress tumor cell growth of epidermoid and colorectal carcinoma-derived cell lines in vitro (Rodeck et al., 1987). Radiolabelled MAb 425 has also
20   been shown to bind to xenografts of human malignant gliomas in mice (Takahashi et al., 1987).

EGF is a polypeptide hormone which is mitogenic for epidermal and epithelial cells. When EGF interacts with sensitive
25   cells, it binds to membrane receptors; the receptor EGF complexes cluster and then are internalized in endocytotic vesicles. This is responsible for the phenomenon of "down-regulation". EGF binding induces a tyrosine kinase activity of the receptor molecule and induces synthesis of DNA.
30

The EGF-receptor is a transmembrane glycoprotein of about
170,000 Daltons (Cohen, 1982). It is the gene product of the
c-erb-B proto-oncogene (Downward et al., Nature, Vol. 307,
pp. 521-527, 1984). The receptor exists in two kinetic forms:
5   so-called low affinity and high-affinity receptors.

The A431 carcinoma cell line expresses abundant EGF-receptors
on its cell surfaces, and thus has been used in many studies
to generate anti-EGF-receptor antibodies. However, the recep-
10   tors on A431 differ from those of other cell types in the
carbohydrate moieties attached to the polypeptide. Thus many
antibodies raised against A431 membranes are directed against
carbohydrates which are not common to all forms of the recep-
tor molecule (e.g. Schreiber, 1983).

15

Other monoclonal antibodies are reactive with the protein
moiety of EGF-receptors. These antibodies display a variety
of properties upon binding to EGF-receptors, presumably
dependent on the particular portion of the receptor molecule
20   bound, and the isotype of the antibody. Some antibodies mimic
some of the effects of EGF (agonists) and some inhibit the
effects (antagonists).

Expression of EGF-receptors has been implicated in the pro-
25   gression of tumor growth. The gene for the receptors has been
found to be the cellular analogue of the avian viral oncogene
v-erb-B (Ulrich, 1984). In addition an association has been
detected between late stages of melanoma development and
extra copies of the chromosome carrying the receptor gene
30   (Koprowski et al., Somatic Cell and Molecular Genetics, Vol.
11, pp. 297-302, 1985).

Because of EGF-receptors are expressed on a wide variety of
solid tumors they provide a suitable target for anti-tumor
therapy. However, there is a need in the art for a suitable
anti-receptor antibody. Many of the known antibodies have
5   properties which would be deleterious if used as anti-tumor
agents. For example, antibodies which mimic the effects of
EGF could stimulate the progression of the tumor rather than
arresting it. Other antibodies which only bind to high or low
affinity receptors could be less than optimally effective
10   because EGF could still exert its effect through the unbound
receptors. Still other antibodies convert low affinity recep-
tors to high affinity receptors, which could exacerbate tumor
growth rather than inhibiting it. Thus there is a need in the
art for an anti-EGF-receptor antibody which would be suitable
15   for anti-tumor therapy.

Although murine MAbs have been used for therapeutic treatment
in humans, they have elicited an immune response (Giorgi et
al., 1983; Jaffers et al., 1986). To overcome this problem,
20   several groups have tried to "humanize" murine antibodies.
This can involve one of two approaches. Firstly, the murine
constant region domains for both the light and heavy chain
can be replaced with human constant regions. Such "chimeric"
murine-human antibodies have been successfully constructed
25   from several murine antibodies directed against human tumor-
associated antigens (Sun et al., 1987; Whittle et al., 1987;
Liu et al., 1987; Gillies and Wesolowski, 1990). This
approach totally conserves the antigen-binding site of the
murine antibody, and hence the antigen affinity, while con-
30   ferring the human isotype and effector functions. In the
second approach only the complementarity determining regions

(CDRs) from the mouse variable regions are grafted together
with human framework regions (FRs) of both the light and
heavy chain variable domains (V_L and V_H). It is reasoned that
this technique will transfer the critical and major portion

5 of the antigen-binding site to the human antibody (Jones et
al., 1986).

CDR grafting has been carried out for several rodent mono-
clonals (Jones et al., 1986; Reichmann et al., 1988; Verhoe-

10 yen et al.; 1988; Queen et al.; 1989; Co et al., 1991; Gorman
et al., 1991; Maeda et al., 1991; Temptest et al., 1991). All
retained their capacity to bind antigen, although the affin-
ity was usually diminished. In most cases it was deemed
necessary to alter certain amino acids in the human framework

15 residues (FRs). Both chimeric and CDR grafted antibodies have
proved superior to the mouse antibodies in the clinic (Hale
et al., 1988; LoBuglio et al., 1989; Mathieson et al., 1990).
However, a general teaching of which amino acids have to be
changed, is not known and not completely predictable in any

20 case.

EP 088 994 proposes the construction of recombinant DNA
vectors comprising of a DNA sequence which codes for a vari-
able domain of a light or a heavy chain of an immunoglobulin

25 specific for a predetermined ligand. The application does not
contemplate variations in the sequence of the variable
domain.

EP 102 634 describes the cloning and expression in bacterial

30 host organisms of genes coding for the whole or a part of
human IgG heavy chain polypeptide, but does not contemplate
variations in the sequence of the polypeptide.

EP 239 400 proposes that humanized antibodies can be obtained by replacing the antigen-binding site (hypervariable regions) of any human antibody by an antigen-binding site of a non-human, for example of a mouse or a rat antibody by genetechno-
5   logical methods.

Thus, following this teaching, human or humanized antibodies can be manufactured having specific antigen-binding sites which were not available up to now in antibodies originating
10   from humans.

Chimeric antibodies can be obtained by replacing not only the CDRs but the whole variable regions of the light and heavy chains. Chimeric antibodies, however, can still be immuno-
15   genic. Chimeric antibodies are, however, very useful for diagnostic purposes and optimizing humanized antibodies.

It could be shown that the affinity of the antigen-binding sites can be influenced by selective exchange of some single
20   amino acids within the variable regions which are not directly part of the CDRs (Reichmann et al., 1988).

As consequence in the worst case, the binding affinity of the antigen can be completely lost if one works according to the
25   teaching of the EP 239 400. This fact could be demonstrated by the inventors of the instant invention, who failed in constructing a correspondingly humanized antibody which was directed to epitopes of the EGF-receptor.

30   Therefore, it must be considered that the success of such a humanization depends on the constitution and conformation of the used variable regions and their interactions with the

corresponding antigen-binding site. Thus, it is not com-
pletely predictable whether or which modifications within the
variable domains of the antibody are necessary in order to
obtain or to improve the binding of the antigen to the anti-
5   body.

SUMMARY OF THE INVENTION

Thus, the invention has the object of providing a humanized
10 monoclonal antibody which is, in particular, directed to the
EGF-receptor, comprising an antigen-binding site of non-human
sources and the FRs of the variable regions and constant
regions of human origins, which are, if necessary, modified
in a way that the specificity of the binding site can be
15 conserved or restored.

In particular, the invention has the object of characterizing
the hypervariable regions of the antigen-binding site of an
antibody against the EGF-receptor and providing these CDRs
20 within a humanized monoclonal antibody defined as above.

This antibody and its chimeric variant can play an important
role as a therapeutic or diagnostic agent in order to combat
tumors, as melanoma, glioma or carcinoma.

25

It has been found, that effective and specific humanized
monoclonal antibodies can be easily obtained by using a
consensus sequence of at least the heavy chain variable
regions of human immunoglobulins. In particular, all those
30 consensus sequences are suitable which have a good (at least
60-70 %, particularly 65-70 %) identity compared with the
variable regions of the original non-human antibodies.

.Furthermore, it has been found, that these consensus sequences have to be modified only to a low extent whereas sometimes much more modifications have to be undertaken using variable regions of naturally occurring human antibodies. Often no or only a few modifications in the amino acid sequence are necessary according to the invention in order to receive a good specific antigen binding. Thus, only a few amino acids must be replaced in getting a perfect binding of the EGF-receptor to the preferred humanized antibody according to the invention, whereas no binding can be obtained here according to the teaching of the EP 239 400. The modifications which are necessary according to the invention can be indicated with 0 to 10 %, or preferably, 1 to 5 % related to the exchange of amino acids.

A humanized monoclonal antibody according to the invention has the following advantage: a consensus sequence which is a sequence according to the most common occurrence of amino acid on a distinct position of a chain of human immunoglobulin of a defined class or subclass or subgroup, can be synthesized as a whole or as a part without problems. There is no dependence on the detailed knowledge or availability of certain individual antibodies or antibody fragments. That means that a wide range of individually and naturally occurring antibody fragments can be covered by providing a very restricted number of consensus sequences which are cloned into corresponding expression vectors. A consensus sequence may be favorable with respect to the immunogenicity in comparison with individual natural sequences which are known to be sometimes epitopes for other antibodies (for example anti-idiotypic antibodies).

Although only one preferred embodiment was made, a general principal teaching is disclosed according to the instant invention. It is not a mere accident with respect to the large number of possible sequences and combinations of

5   sequences in the variable and hypervariable domains that the described teaching regarding the consensus sequence succeeded in constructing a humanized antibody directed to the EGF-receptor.

10   Furthermore, it has been found, that the heavy chains of the variable domains provide a greater contribution to the antigen-binding site than the corresponding light chains. Therefore, it is not necessary to modify in the same manner the light chain of a humanized antibody having a consensus

15   sequence. This is an interesting aspect because it is known that the light chains in some known natural antibodies play the more important role than the corresponding heavy chains (see Williams et al., 1990).

20   Finally and above all, the invention provides for the first time the characterization, cloning and amplification by means of genetic engineering the antigen-binding site of a murine antibody against the EGF-receptor (MAb 425). Corresponding oligonucleotides could be synthesized which code for that

25   antigen-binding site and for the whole variable domain of a humanized and chimeric monoclonal antibody. The invention provides, moreover, correspondingly effective expression vectors which can be used for the transformation of suitable eukaryotic cells.

30

Thus, the invention relates to a humanized monoclonal anti-body comprising antigen bindings sites (CDRs) of non-human origin, and the FRs of variable regions and constant regions of light and heavy chains of human origin, characterized in that at least the FRs of the variable regions of the heavy chain comprise a modified consensus sequence of different variable regions of a distinct class or subgroup of a human immunoglobulin.

In particular, the invention relates to a humanized mono-clonal antibody, wherein the FRs of the consensus sequence has a homology of at least 70 % compared with the amino acid sequence of the FRs of the variable region of the non-human antibody from which the antigen-binding sites originate.

In particular, the invention relates to a humanized mono-clonal antibody, having the following properties:

(a) binds to human EGF-receptors;

(b) inhibits binding of EGF to EGF-receptor;

(c) inhibits the EGF-dependent tyrosine kinase activity of EGF-receptor;

(d) inhibits the growth of EGF-sensitive cells.

In particular, the invention relates to a humanized mono-clonal antibody, wherein the hypervariable regions of the antigen-binding sites comprise the following amino acid sequences:

light chain

      CDR-1   -Ser-Ala-Ser-Ser-Ser-Val-Thr-Tyr-Met-Tyr-
      CDR-2   -Asp-Thr-Ser-Asn-Leu-Ala-Ser-
5   CDR-3   -Gln-Gln-Trp-Ser-Ser-His-Ile-Phe-Thr-

heavy chain

      CDR-1   -Ser-His-Trp-Met-His-
10   CDR-2   -Glu-Phe-Asn-Pro-Ser-Asn-Gly-Arg-Thr-Asn-Tyr-Asn-Glu-
                 Lys-Phe-Lys-Ser-
      CDR-3   -Arg-Asp-Tyr-Asp-Tyr-Asp-Gly-Arg-Tyr-Phe-Asp-Tyr-

In particular, the invention relates to a humanized mono-
15 clonal antibody, wherein the FRs of the variable regions
which are not related to the antigen-binding sites comprise
the following amino acid sequence:

light chain

20

      FR-1   -Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-Ala-
                 Ser-Val-Gly-Asp-Arg-Val-Thr-Ile-Thr-Cys-
      FR-2   -Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Lys-Ala-Pro-Lys-Leu-Leu-
                 Ile-Tyr-
25   FR-3   -Gly-Val-Pro-Ser-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-Thr-
                 Asp-Tyr(Phe,Trp,His)-Thr-Phe-Thr-Ile-Ser-Ser-Leu-Gln-
                 Pro-Glu-Asp-Ile-Ala-Thr-Tyr-Tyr-Cys-
      FR-4   -Phe-Gly-Gln-Gly-Thr-Lys-Val-Glu-Ile-Lys-

30

heavy chain

FR-1   -Gln-Val-Gln-Leu-Val-Gln-Ser-Gly-Ala-Glu-Val-Lys-Lys-
          Pro-Gly-Ala-Ser-Val-Lys-Val-Ser-Cys-Lys-Ala-Ser-Gly-
          Tyr-Thr-Phe-Thr(Ser)-

FR-2   -Trp-Val-Arg(His)-Gln-Ala(Lys,His)-Pro(Val)-Gly-Gln-
          Gly-Leu-Glu-Trp-Ile(Val,Leu)-Gly-

FR-3   -Lys(Arg,His)-Ala(Val,Pro-Gly)-Thr-Met-Thr-
          Val(Ala,Pro,Gly)-Asp-Thr-Ser-Thr-Asn-Thr-Ala-Tyr-Met-
          Glu(Asn)-Leu-Ser-Ser-Leu-Arg-Ser-Glu-Asp-Thr-Ala-Val-
          Tyr-Tyr-Cys-Ala-Ser-

FR-4   -Trp-Gly-Gln-Gly-Thr-Leu-Val-Thr-Val-Ser-Ser-,

and wherein the amino acids listed in the brackets are alter-
natives.

In particular, the invention relates to a humanized mono-
clonal antibody, wherein the constant regions of the heavy
chain comprise the amino acid sequence of a gamma-1 chain,
and the constant regions of the light chain comprise the
amino acid sequence of a kappa chain of a human immunoglobu-
lin.

In particular, the invention relates to a humanized mono-
clonal antibody, comprising a derivate of an amino acid
sequence modified by amino acid deletion, substitution,
addition or inversion within the variable and constant
regions wherein the biological function of specific binding
to the antigen is preserved.

Furthermore, the invention relates to an expression vector,
suitable for transformation of host cells, characterized in
that it comprises a DNA sequence coding for the variable
and/or constant regions of the light and/or heavy chains of a
5 humanized antibody.

Furthermore, the invention relates to humanized or chimeric
monoclonal antibody, comprising hypervariable regions (CDRs)
of antigen-binding sites of murine origin and the FRs of the
10 variable regions of human or murine origin and constant
regions of light and heavy chains of human origin, character-
ized in that the hypervariable regions comprise the following
amino acid sequences,

15 **light chain**

CDR-1   -Ser-Ala-Ser-Ser-Ser-Val-Thr-Tyr-Met-Tyr-
CDR-2   -Asp-Thr-Ser-Asn-Leu-Ala-Ser-
CDR-3   -Gln-Gln-Trp-Ser-Ser-His-Ile-Phe-Thr-

20
heavy chain

CDR-1   -Ser-His-Trp-Met-His-
CDR-2   -Glu-Phe-Asn-Pro-Ser-Asn-Gly-Arg-Thr-Asn-Tyr-Asn-Glu-
25   Lys-Phe-Lys-Ser-
CDR-3   -Arg-Asp-Tyr-Asp-Tyr-Asp-Gly-Arg-Tyr-Phe-Asp-Tyr-,

and wherein the constant regions of the heavy chain comprise
the amino acid sequence of a gamma-1 chain, and the constant
30 regions of the light chain comprise the amino acid sequence
of a kappa chain of a human immunoglobulin.

In particular, the invention relates to a humanized mono-
clonal antibody according to claim 12, wherein the FRs of the
variable regions which are not related to the antigen-binding
sites, are of human origin and comprise the following amino
5   acid sequence,

light chain

         FR-1   -Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-Ala-
10   Ser-Val-Gly-Asp-Arg-Val-Thr-Ile-Thr-Cys-
         FR-2   -Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Lys-Ala-Pro-Lys-Leu-Leu-
                   Ile-Tyr-
         FR-3   Gly-Val-Pro-Ser-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-Thr-
                   Asp-Tyr(Phe,Trp,His)-Thr-Phe-Thr-Ile-Ser-Ser-Leu-Gln-
15   Pro-Glu-Asp-Ile-Ala-Thr-Tyr-Tyr-Cys-
         FR-4   -Phe-Gly-Gln-Gly-Thr-Lys-Val-Glu-Ile-Lys-

heavy chain

20   FR-1   -Gln-Val-Gln-Leu-Val-Gln-Ser-Gly-Ala-Glu-Val-Lys-Lys-
                   Pro-Gly-Ala-Ser-Val-Lys-Val-Ser-Cys-Lys-Ala-Ser-Gly-
                   Tyr-Thr-Phe-Thr(Ser)-
         FR-2   -Trp-Val-Arg(His)-Gln-Ala(Lys,His)-Pro(Val)-Gly-Gln-
                   Gly-Leu-Glu-Trp-Ile(Val,Leu)-Gly-
25   FR-3   -Lys(Arg,His)-Ala(Val,Pro,Gly)-Thr-Met-Thr-
                   Val(Ala,Pro,Gly)-Asp-Thr-Ser-Thr-Asn-Thr-Ala-Tyr-Met-
                   Glu(Asn)-Leu-Ser-Ser-Leu-Arg-Ser-Glu-Asp-Thr-Ala-Val-
                   Tyr-Tyr-Cys-Ala-Ser-
         FR-4   -Trp-Gly-Gln-Gly-Thr-Leu-Val-Thr-Val-Ser-Ser-

30

In particular, the invention relates to a chimeric monoclonal antibody according to Claim 12, wherein the FRs of the variable regions which are not related to the antigen-binding site, are of murine origin and comprise the following amino acid sequences:

light chain

FR-1   -Gln-Ile-Val-Leu-Thr-Gln-Ser-Pro-Ala-Ile-Met-Ser-Ala-
        Ser-Pro-Gly-Glu-Lys-Val-Thr-Met-Thr-Cys-

FR-2   -Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Ser-Ser-Pro-Arg-Leu-Leu-
        Ile-Tyr-

FR-3   -Gly-Val-Pro-Val-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-Thr-
        Ser-Tyr-Ser-Leu-Thr-Ile-Ser-Arg-Met-Glu-Ala-Glu-Asp-
        Ala-Ala-Thr-Tyr-Tyr-Cys-

FR-4   -Phe-Gly-Ser-Gly-Thr-Lys-Leu-Glu-Ile-Lys-

heavy chain

FR-1   -Gln-Val-Gln-Leu-Gln-Gln-Pro-Gly-Ala-Glu-Leu-Val-Lys-
        Pro-Gly-Ala-Ser-Val-Lys-Leu-Ser-Cys-Lys-Ala-Ser-Gly-
        Tyr-Thr-Phe-Thr-

FR-2   -Trp-Val-Lys-Gln-Arg-Ala-Gly-Gln-Gly-Leu-Glu-Trp-Ile-
        Gly-

FR-3   -Lys-Ala-Thr-Leu-Thr-Val-Asp-Lys-Ser-Ser-Ser-Thr-Ala-
        Tyr-Met-Gln-Leu-Ser-Ser-Leu-Thr-Ser-Glu-Asp-Ser-Ala-
        Val-Tyr-Tyr-Cys-Ala-Ser-

FR-4   -Trp-Gly-Gln-Gly-Thr-Thr-Leu-Thr-Val-Ser-Ser-

Moreover, the invention relates to an expression vector, suitable for transformation of host cells, characterized in that it comprises DNA sequences coding for the variable and/or constant regions of the light and/or heavy chains of a humanized or chimeric monoclonal antibody.

Furthermore, the invention relates to a process for the preparation of a humanized monoclonal antibody, comprising hypervariable regions (CDRs) of antigen-binding sites of non-human origin, and FRs of variable regions and constant regions of the light and heavy chains of human origin by cultivating transformed host cells in a culture medium and purification and isolation the expressed antibody proteins, characterized in

(a) synthesizing or partially synthesizing or isolating an oligonucleotide sequence which codes for an amino acid consensus sequence of different variable regions (FR-1 to FR-4) of a heavy chain of a class or a subgroup of a human immunoglobulin, wherein the used consensus sequence has a homology of at least 70 % compared with the amino acid sequence of the FRs of the variable regions of the non-human antibody from which the antigen-binding sites originate, and wherein the consensus sequence is modified by alterations of maximum 10 % of the amino acids in order to preserve the binding capability of the antigen to the hypervariable regions;

(b) synthesizing or partially synthesizing or isolating an oligonucleotide sequence which codes for an amino acid consensus sequence under the conditions given in (a) of

different variable regions (FR-1 to FR-4) of a light
chain of a class or a subgroup of a human immunoglobulin,
or, alternatively, which codes for a corresponding natu-
ral occurring amino acid sequence;

5

(c) in each case synthesizing or partially synthesizing or
isolating an oligonucleotide sequence which codes for the
amino acid sequence of the hypervariable regions (CDRs)
of the light and heavy chain corresponding to the hyper-
variable regions of the basic non-human antibody;

10

(d) in each case synthesizing or partially synthesizing or
isolating an oligonucleotide sequence which codes for the
amino acid sequence of the constant regions of the light
and heavy chain of a human immunoglobulin;

15

(e) constructing one or several expression vectors comprising
in each case at least a promoter, a replication origin
and the coding DNA sequences according to (a) to (d),
wherein the DNA sequences coding for the light and heavy
chains can be present together in one or, alternatively,
in two or more different vectors,

20

and finally,

25

(f) transforming the host cells with one or more of the
expression vectors according to (e).

In particular, the invention relates to a process, wherein
DNA sequences are used coding for the following amino acid
sequences which represent the hypervariable regions (CDRs):

30

light chain

     CDR-1   -Ser-Ala-Ser-Ser-Ser-Val-Thr-Tyr-Met-Tyr-
     CDR-2   -Asp-Thr-Ser-Asn-Leu-Ala-Ser-
5   CDR-3   -Gln-Gln-Trp-Ser-Ser-His-Ile-Phe-Thr-

heavy chain

     CDR-1   -Ser-His-Trp-Met-His-
10   CDR-2   -Glu-Phe-Asn-Pro-Ser-Asn-Gly-Arg-Thr-Asn-Tyr-Asn-Glu-
               Lys-Phe-Lys-Ser-
     CDR-3   -Arg-Asp-Tyr-Asp-Tyr-Asp-Gly-Arg-Tyr-Phe-Asp-Tyr-

In particular, the invention relates to a process, wherein
15   DNA sequences are used coding for the following amino acid
     sequences which represent the FRs of the variable regions :

light chain

20   FR-1   -Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-Ala-
               Ser-Val-Gly-Asp-Arg-Val-Thr-Ile-Thr-Cys-
     FR-2   -Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Lys-Ala-Pro-Lys-Leu-Leu-
               Ile-Tyr-
     FR-3   -Gly-Val-Pro-Ser-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-Thr-
25   Asp-Tyr(Phe,Trp,His)-Thr-Phe-Thr-Ile-Ser-Ser-Leu-Gln-
               Pro-Glu-Asp-Ile-Ala-Thr-Tyr-Tyr-Cys-
     FR-4   -Phe-Gly-Gln-Gly-Thr-Lys-Val-Glu-Ile-Lys-

30

heavy chain

    FR-1   -Gln-Val-Gln-Leu-Val-Gln-Ser-Gly-Ala-Glu-Val-Lys-Lys-
              Pro-Gly-Ala-Ser-Val-Lys-Val-Ser-Cys-Lys-Ala-Ser-Gly-
5   Tyr-Thr-Phe-Thr(Ser)-
    FR-2   -Trp-Val-Arg(His)-Gln-Ala(Lys,His)-Pro(Val)-Gly-Gln-
              Gly-Leu-Glu-Trp-Ile(Val,Leu)-Gly-
    FR-3   -Lys(Arg,His)-Ala(Val,Pro,Gly)-Thr-Met-Thr-
              Val(Ala,Pro,Gly)-Asp-Thr-Ser-Thr-Asn-Thr-Ala-Tyr-Met-
10   Glu(Asn)-Leu-Ser-Ser-Leu-Arg-Ser-Glu-Asp-Thr-Ala-Val-
              Tyr-Tyr-Cys-Ala-Ser-
    FR-4   -Trp-Gly-Gln-Gly-Thr-Leu-Val-Thr-Val-Ser-Ser

Moreover, the invention relates to a process for the prepara-
15 tion of a chimeric monoclonal antibody having the biological
function of binding to epitopes of the EGF-receptor, compris-
ing hypervariable regions (CDRs) of antigen-binding sites and
FRs of variable regions of murine origin   and FRs of
variable regions of murine origin and constant regions of the
20 light and heavy chains of human origin by cultivating trans-
formed host cells in a culture medium and purification and
isolation the expressed antibody proteins, characterized in
that the host cells are transformed with expression vectors
according to one of the expression vectors.

25

Furthermore, the invention relates to a pharmaceutical compo-
sition comprising a humanized or chimeric monoclonal anti-
body.

30 Furthermore, the invention relates to the use of humanized or
chimeric antibody for the manufacture of a medicament
directed to tumors.

Finally, the invention relates to the use of humanized or chimeric antibody for diagnostic locating and assessing tumor growth.

5   To sum up, the invention relates to a monoclonal antibody comprising a consensus sequence of variable regions of a heavy chain of a class or a subgroup of human immunoglobulins.

10   The entire disclosures of all applications, patents and publications, if any, cited above and below, and of corresponding European Patent application 91 103 389.2, filed March 6, 1991, are hereby incorporated by reference.

15   <u>Microorganisms and plasmids used in the invention:</u>

(a) **pRVL425 (= HCMV-RV$_L$b425-k)**, deposited on February 1, 1991, according to the Budapest Treaty at the Deutsche Sammlung von Mikroorganismen (DSM) under the <u>accession No.</u>
20   <u>DMS 6340</u>. The expression vector contains the sequences of the hypervariable regions (CDRs) of the murine antibody 425 and the FRs of the variable region and the constant (kappa) region of the light chain of the humanized antibody. R is standing for "reshaped".

25

(b) **pRVH425 (= HCMV-RV$_H$g425-γ)**, deposited on February 1, 1991, according to the Budapest Treaty at the Deutsche Sammlung von Mikroorganismen (DSM) under the <u>accession No.</u>
      <u>DSM 6339</u>. The expression vector contains the sequences of
30   the hypervariable regions (CDRs) of the murine antibody

425 and the FRs of variable region and constant (gamma-1) region of the heavy chain of the humanized antibody. R is standing for "reshaped".

(c) **pCVL425 (= HCMV-CV$_L$425-k)**, deposited on February 1, 1991, according to the Budapest Treaty at the Deutsche Sammlung von Mikroorganismen (DSM) under the accession No. DSM 6338. The expression vector contains the sequences of the FRs and hypervariable regions (CDRs) of the light chain variable region of the murine antibody 425 and the constant (kappa) region of the light chain of human immunoglobulin. C is standing for chimeric.

(d) **pCVH425 (= HCMV-CV$_H$425-γ)**, deposited on February 1, 1991, according to the Budapest Treaty at the Deutsche Sammlung von Mikroorganismen (DSM) under the accession No. DSM 6337. The expression vector contains the sequences of the FRs and hypervariable regions (CDRs) of the light chain variable region of the murine antibody 425 and the constant region of the light chain of the human gamma-1 immunoglobulin. C is standing for chimeric.

(e) **Hybridoma cell line 425**, deposited on January 26, 1988, according to Budapest Treaty at the American Type Culture Collection (ATCC) under the accession No. HB 9629. The cell line produces the murine antibody 425 which is directed to the EGF-receptor.

Other biological materials:

Other microorganisms, cell lines, plasmids, promoters, resistance markers, replication origins or other fragments of
vectors which are mentioned in the application are commercially or otherwise generally available. Provided that no
other hints in the application are given, they are used only
as examples and are not essential according to the invention
and can be replaced by other suitable tools and biological
materials, respectively.

Bacterial hosts are preferably used for the amplification of
the corresponding DNA sequences. Examples for these host are:
E. coli or Bacillus.

Eukaryotic cells like COS (CV1 origin SV40) or CHO (Chinese
hamster ovary) cells or yeasts, for example, are preferred in
order to produce the humanized and chimeric antibodies
according to the invention. COS and CHO cells are preferred.

General methods for manufacturing:

The techniques which are essential according to the invention
are described in detail in the specification.

Other techniques which are not described in detail correspond
to known standard methods which are well known to a person
skilled in the art or are described more in detail in the
cited references and patent applications and in standard
literature.

Brief descriptions of the Figures

Fig. 1   Schematic representations of the vectors used for the
          expression of chimeric and reshaped human antibodies.
5   Restriction sites used in the construction of the
          expression plasmids are marked. The variable region
          coding sequences are represented by the dark boxes,
          constant regions by the light boxes, the HCMV pro-
          moter and enhancer by the hatched boxes, and the
10   nucleotide fragment from the plasmid pSVneo by the
          speckled boxes. The directions of transcription are
          represented by arrows.

Fig. 2   The nucleotide and amino acid sequences of the $V_H 425$
15   (A), and $V_L 425$ (B) cDNA as cloned into pUC18. The
          amino acids contributing to the leader are underlined
          and CDRs are indicated by brackets. The splice sites
          between the variable regions and constant regions are
          also shown. The front and back PCR-primers and their
20   annealing sites, used in the construction of the
          genes coding for the chimeric antibodies, are shown.

Fig. 3   The nucleotide and amino acid sequences of the syn-
          thesized gene fragment coding for reshaped human
25        $V_H a 425$. The leader sequence is underlined and resi-
          dues contributing to the CDRs are bracketed.

Fig. 4   Comparison of the amino acid sequences of mouse and
          reshaped human 425 variable regions. Panel A shows
30   the sequences of mouse V_L ($V_L 425$) and reshaped human
          $V_L S$ ($RV_L a 425$ and $RV_L b 425$). Panel B shows the sequences

of mouse V_H (V_H425) and reshaped human V_Hs (RV$_H$a425, RV$_H$b425, RV$_H$c425, RV$_H$d425, RV$_H$e425, RV$_H$f425, RV$_H$g425, RV$_H$h425, and RV$_H$i425). The FRs and CDRs are indicated. Amino acids are numbered according to Kabat et al., 1987.

Fig. 5 Molecular model of the mouse MAb 425 variable regions.

Fig. 6 Detection of binding to EGFR by ELISA. Antigen-binding activity was assayed in dilutions of transfected COS cell supernatants and plotted as optical density at 450 nm against concentration of IgG (quantitated by ELISA, see Materials and Methods). All versions of reshaped human V_H regions were cotransfected with RV$_L$a425 and are represented as follows: RV$_H$a425 ▲, RV$_H$b425 ◊, RV$_H$c425 Δ, RV$_H$d425 ⊗, RV$_H$e425 □, RV$_H$f425 ⊠, RF$_H$g425 □, RV$_H$h425 ○, RV$_H$i425 ⊙, RV$_H$b425 co-transfected with RV$_L$b425 is represented as ◆. A co-transfection of the chimeric VL425 and VH425 are represented as ●.

Fig. 7 Competition for binding to antigen. Panel A shows competition between labelled mouse 425 antibody and (1) unlabelled mouse 425 antibody (+) and (2) chimeric 425 antibody (●) produced by COS cells after co-transfection with HCMV-CV$_L$425-kappa and HCMV-C$_H$425-gamma-1. Panel B shows competition between labelled mouse 425 antibody and (1) unlabelled mouse 425 antibody (+) and (2) the reshaped human 425 antibodies

produced by COS cells after co-transfection with
HCMV-RV$_L$a425-kappa and HCMV-RV$_H$i425-gamma-1 (o), and
with HCMV-RV$_L$a425-kappa and HCMV-RV$_H$g425-gamma-1 (□).

5   In each case, the horizontal axis represents the
concentration of inhibitor (ng/ml). The vertical axis
represents percentage of inhibition of binding.

Fig. 8   An examination of the effects of different reshaped
human V$_L$ regions on antigen-binding. Panel A shows

10   antigen-binding by reshaped human antibodies produced
in COS cells transfected with HCMV-CV$_L$425-kappa and
HCMV-CV$_H$425-gamma-1 (●), HCMV-RV$_L$a425-kappa and HCMV-
RV$_H$g425-gamma-1 (□), HCMV-RV$_L$b425-kappa and HCMV-

15   RV$_H$g425-gamma-1 (■), HCMV-RV$_L$a425-kappa and HCMV-
RV$_H$c425-gamma-1 (Δ), and HCMV-RV$_L$b425-kappa and HCMV-
RV$_H$c425-gamma-1 (▲). Panel B shows competition for
binding to antigen between labelled mouse 425 anti-
body and (1) unlabelled mouse 425 antibody (+) and

20   (2) reshaped human 425 antibodies produced in COS
cells co-transfected with HCMV-V$_L$a425-kappa and HCMV-
V$_H$g425-gamma-1 (□) and with HCMV-V$_L$b425-kappa and
HCMV-V$_H$g425-gamma-1 (■). In panel A, the vertical

25   axis represents the optical density at 450 nm (OD$_{450}$)
and the horizontal axis represents the concentration
of IgG (ng/ml). In panel B, the horizontal axis rep-
resents the concentration of inhibitor (ng/ml) and
the vertical axis represents percentage of inhibition

30   of binding.

Fig. 9   Panel A: Analysis of reshaped (lanes 1, 2), chimeric
(lane 3) and murine (lane 4) MAbs 425 by SDS-PAGE
under non-reducing conditions (a) and under reducing
conditions (b). Reshaped (lanes 7, 8), chimeric (lane
5   9) and murine (lane 10). Lanes 5, 6, 11, and 12 are
MW markers.

Panel B: Purification by gel filtration of reshaped
MAb 425 on Superose 12. Peak 2 represents IgG.

10

Fig. 10   Competitive binding of murine, chimeric and reshaped
MAbs 425 to EGF-receptor (EGFR). The vertical axis
represents the ratio bound (MAb) to total (MAb) in %
(% bound/total). The horizontal axis represents the
15   concentration of antibody (mol/l [log]).

∇ means MAb 425 murine

ο means MAb 425 chimeric

●, ▼ mean MAb 425 reshaped

20

Fig. 11   Competition of EGF and antibodies to EGF-receptor.
The vertical axis represents % bound/total (MAb). The
horizontal axis represents the concentration of anti-
body (mol/l [log]).

25

ο means MAb 425 murine

Δ, ∇, ▢ mean MAb 425 reshaped

30

DETAILED DESCRIPTION

Cloning and sequencing of variable region genes of MAb 425:

5   From the cDNA synthesis and cloning using the kappa chain
      primer, 300-400 colonies are preferably picked for screening.
      From the cDNA synthesis and cloning using the gamma-2a
      primer, 200-300 colonies are preferably for screening. After
      screening by hybridization using the two respective cloning
10   primers, 20-30 light chain colonies and 10-20 heavy chain
      colonies give strong signals. Plasmid DNA is isolated from
      these colonies and analyzed by usual and commercially avail-
      able restriction enzyme digests to determine the size of the
      cDNA inserts. Clones that appear to have inserts 400-500 bp
15   or 500-600 bp for V_L and V_H cloning, respectively, are
      selected as candidates for sequencing. Three V_L clones and
      three V_H clones are sequenced on both strands using M13
      universal and reverse sequencing primers. Of the three possi-
      ble V_L clones sequenced, one codes for a complete variable
20   region and the others appears to code for unrelated peptides.
      Two of the V_H clones code for identical V_H regions while the
      other appears to code for the V_H region with the intron
      between the leader sequence and FR-1 still present. Apart
      from the intron, the third V_H clone contains coding sequence
25   identical to that of the first two clones. To verify the
      sequence of the V_L region, three more cDNA clones containing
      inserts of the appropriate size are sequenced. Two of these
      give sequences in agreement with the first V_L clone. The third
30   is an unrelated DNA sequence. In the clones sequenced, not

all of the original primer sequence are present. The extent
of the deletions varies from clone to clone. These deletions,
which probably occur during cDNA synthesis and cloning, may
decrease the efficiency of the colony screening.

5

The V_L and V_H genes for MAb 425 are shown in Figure 2. The
amino acid sequence of the 425 V_L and V_H regions, are compared
to other mouse variable regions in the Kabat data base (Kabat
et al., 1987). The V_L region can be classified into the mouse

10 kappa chain variable region subgroup IV or VI. Within the
FRs, the 425 V_L region has an approximately 86 % identity to
the consensus sequence for mouse kappa subgroup IV and an
approximately 89 % identity to subgroup VI. The 425 V_L region
appear to use the JK4 segment. Examination of the VH region

15 shows an approximately 98 % identity to the FRs of the con-
sensus sequence for mouse heavy chain subgroup II (B).

The right choice of a suitable class or subgroup of human
immunoglobulin is dependent on the extent of the identity to

20 the originally present chain in the non-human antibody. The
identity of the deduced consensus sequence according to the
present invention should be greater than 65 to 70 % compared
with the sequence of the original non-human chain.

25

The consensus sequences of the heavy chains are preferred
especially, however, the consensus sequence of human heavy
chain subgroup I. However, for other antibodies, the consen-
sus sequences of other human heavy chains are suitable. The
preferred consensus sequences are modified. The possible

30 exchange of amino acids is 0 to 10 % according to the inven-
tion, preferably 5 to 10 %.

Construction and expression of chimeric 425 antibody:

Before the cDNAs coding for the VL and VH regions can be used
in the construction of chimeric 425 antibody , it is neces-
sary to introduce several modifications at the 5'- and
3'ends. these include introducing appropriate restriction
enzyme sites so that the variable region coding sequences can
be conveniently subcloned into the HCMV expression vectors.
It is necessary to re-create donor splice sites in the 3'-
flanking regions so that the variable regions are spliced
correctly and efficiently to the constant regions. The 5'-
flanking regions are also modified to include a sequence that
would create efficient initiation sites for translation by
eukaryotic ribosomes (Kozak, 1987). These modifications are
introduced using PCR primers. The used primers are indicated
in Table 1.

Table 1 Oligonucleotides used for cDNA cloning, construction
of chimerics, and mutagenesis. Underlined sections
denote bases that anneal to the human framework.

Number	Sequence	Description
1.	5'-GTAGGATCCTGGATGGTGGGAAGATG-3'	Light chain primer for cDNA synthesis.
2.	5'-GTAGGATCCAGTGGATAGACCGATG-3'	Heavy chain primer for cDNA synthesis.
3.	5'-CTCCAAGCTTGACCTCACCATGG-3'	Chimeric V_H front primer.

Number	Sequence	Description
4.	5'-TTGGATCCACTCACCTGAGGAGACTGTGA-3'	Chimeric V_H back primer.
5.	5'-AGAAAGCTTCCACCATGGATTTTCAAGTG-3'	Chimeric V_L front primer.
6.	5'-GTAGATCTACTCACGTTTTATTTCCAAC-3'	Chimeric V_L back primer.
7.	5'-<u>ACCATCACCTGT</u>AGTGCCAGCTCAAGTG TAACTTACATGTAT<u>TGGTACCAGCAG</u>-3'	Reshaped V_L CDR-1 primer.
8.	5'-<u>CTGCTGATCTAC</u>GACACATCCAACCTGGC TTCT<u>GGTGTGCCAAGC</u>-3'	Resphaped V_L CDR-2 primer.
9.	5'-<u>ACCTACTACTGC</u>CAGCAGTGGAGTAGTCA-CATATTC<u>ACGTTCGGCCAA</u>-3'	Resphaped V_L CDR-3 primer.
10.	* 5'-AGCGGTACCGACTACACCTTCACCATC-3'	Primer to introduce F71Y into RV_L.
11.	* * 5'-ATACCTTCACATCCCACTG-3'	Primer to introduce S30T into RV_H.
12.	* * 5'-CGAGTGGATTGGCGAGT-3'	Primer to introduce V48I into RV_H.

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Number	Sequence	Description
	* * *	
13.	5'-TTTAAGAGCAAGGCTACCATGACCGTGGA-CACCTCT-3'	Primer to intro-duce R66K, V67A, L71V into RV_H.
	*	
14.	5'-CATGACCGTGGACACCTCT-3'	Primer to intro-duce L71V into RV_H.

For each variable region cDNA two primers are preferably
designed. In the front primers, 15 bases at the 3'-end of the
primer are used to hybridize the primer to the template DNA
while the 5'-end of the primer contains a HindIII site and
the "Kozak" sequence. The back primers have a similar design
with 15 bases at the 3'-end used to hybridize the primer to
the template DNA and the 5'-end of the primer contains a
BamHI site and a donor splice site. In the case of the light
chain back primer, a BglII site is used instead of BamHI site
because the cDNA coding for the V_L contains an internal BamHI
site (Figure 2). The PCR reaction is preferably carried out
as described in the examples.

The PCR-modified V_L region DNA is cloned into the HindIII-
BamHI sites of the HCMV light chain expression vector as a
HindIII-BglII fragment. This vector already contains the
human genomic kappa constant region with the necessary splice
acceptor site and poly(A^+) sites. The entire PCR-modified V_L
fragment is sequenced using two primers that anneal to sites
flanking the cloning site in the expression vector. Sequenc-
ing confirms that no errors have been incorporated during the

PCR step. The PCR-modified V_H DNA is cloned into the HCMV
heavy chain expression vector as a HindIII-BamHI fragment and
also sequenced to confirm the absence of PCR errors. A BamHI
fragment containing the human genomic gamma-1 constant region

5   is inserted into the HCMV-CV$_H$ vector on the 3'-side of the V_H
region. This fragment contains the necessary acceptor splice
site for the V-C splice to occur in vivo and the naturally
occurring poly(A$^+$) site.

10   The expression vectors containing the chimeric 425 V_L and V_H
regions are co-transfected into appropriate eukaryotic cells,
preferably COS cells. After approximately 72 h of transient
expression, the cell culture medium is assayed by ELISA for
human IgG production and for binding to EGFR protein. Amounts

15   of human IgG detected in the media vary from 100-400 ng/ml.
The chimeric antibody produced binds well to EGFR protein in
a standard antigen-binding ELISA thus confirming that the
correct mouse variable regions has been cloned and sequenced.

20   <u>Initial design, construction and expression or reshaped human
425 light and heavy chains:</u>

In designing a reshaped human 425 antibody, most emphasis is
placed on the V_H region since this domain is often the most

25   important in antigen-binding (Amit et al., 1986; Verhoeyen et
al., 1988). To select the human FRs on which to graft the
mouse CDRs, the FRs of mouse MAb 425 V_H region are compared
with the FRs from the consensus sequences for all subgroups

30

of human V_H regions (Kabat et al., 1987). This comparison shows that the FRs of mouse MAb 425 V_H are most like the FRs of human V_H subgroup I showing an approximately 73 % identity within the FRs and an approximately 65 % identity over the entire V_H regions.

A further comparison of the mouse 425 V_H region with other mouse V_H regions from the same Kabat subgroups is carried out to identity any FR residues which are characteristic of MAb 425 and may, therefore, be involved in antigen binding. The residue at position 94 of the mouse MAb 425 V_H region is a serine while in other V_H regions from mouse subgroup II (B), and also from human subgroup I, residue 94 is an arginine (Kabat et al., 1987). This amino acid substitution is an unusual one and, since position 94 is adjacent to CDR-3, it is at a surprisingly important position. For these reasons, the reshaped human 425 V_H region is preferably designed based on the CDRs of mouse MAb 425 and FRs derived from the consensus sequence for human subgroup I FRs (as defined by Kabat et al., 1987). Positions 94 in FR-3 is made a serine as found in mouse MAb 425. At positions in the consensus sequence for human subgroup I FRs where no single amino acid are listed, the most commonly occurring amino acid at that position is selected. If there is no preferred amino acid at a particular position in the human consensus sequence, the amino acid that is found at that position in the sequence of mouse MAb 425 V_H is selected. The resulting amino acid sequence comprises the first version (versions "a") of reshaped human 425 V_H (Figure 3). All subsequent versions of reshaped human 425 V_H are modifications of this first version.

A 454 bp DNA fragment coding for the reshaped human 425 V_H region, as described above, is designed and synthesized (see examples and Figure 3). In addition to DNA sequences coding for the amino acids of reshaped human 425 V_H region, this DNA

5   fragment also contains sequences coding for a human leader sequence. The human leader sequence can be taken for example from antibody HG3 CL (Rechavi et al., 1983), a member of human V_H subgroup I (Kabat et al., 1987). The synthetic DNA fragment also contains eukaryontic translation signals at the

10   5'-end (Kozak, 1987), a donor splice site at the 3'-end (Breathnach et al., 1978), and HindIII and BamHI sites at the 5'- and 3'-ends, respectively, for subcloning into the HCMV expression vector.

15   A similar procedure is carried out for the design of the reshaped human 425 V_L region. The FRs of mouse MAb 425 V_L region are compared with the consensus sequences for all the subgroups of human V_L regions (Kabat et al., 1987). Within the FRs, an approximately 71 % identity is found between mouse '

20   425 V_L and human kappa V_L subgroup III, and an approximately 70 % identity with human kappa V_L subgroup I. DNA coding for human FRs of human kappa V_L subgroup I is already available from the reshaped human D1.3 V_L region (EP 239 400, Winter)

25   and reshaped human CAMPATH-1 (Reichmann et al., 1988). The design of the reshaped human V_L regions in these two human antibodies is based on the structurally-solved human immuno-globulin REI protein (Epp et al., 1975). For these reasons, the human V_L FRs from reshaped human D1.3 and CAMPATH-1H are

30   also used in reshaped human 425 V_L. A comparison of the FRs of mouse 425 V_L region with FRs of other mouse antibodies from

similar subgroups reveal no significant differences in amino
acid residues at functionally important positions. No changes
in the human FRs are necessary therefore. The amino acid
sequence of the reshaped human 425 V_L region version "a" is

5   shown in Figure 4.

To construct the reshaped human 425 V_L region, three oligonu-
cleotides are designed that contain internal DNA sequences
coding for the three CDRs of mouse 425 V_L region and also

10   contain 12 bases at the 5'- and 3'-ends designed to hybridize
to the DNA sequences coding for the human FRs in reshaped
human D1.3 V_L region (see oligonucleotides 7-9 in Table I).
CDR-grafting is carried as described in the examples. After
DNA sequencing of putative positive clones from the screen-

15   ing, the overall yield of the triple mutant is 5-15 %, pref-
erably 9-10 %. A reshaped human 425 V_L region containing no
PCR errors is cloned as a HindIII-BamHI fragment into the
light chain expression vector to create the plasmid HCMV-
RV_La425-kappa (Figure 1).

20

The two expression vectors bearing the reshaped human 425 V_L
and V_H regions are now co-transfected into appropriate cells
(see above) to look for transient expression of a functional

25   reshaped human 425 antibody. After approximately 72 h, the
cell supernatants are harvested and assayed by ELISA for
human IgG. Human IgG can be detected at levels ranging from
100-500 ng/ml, however, in the ELISA assay for antigen bind-
ing, binding to EGFR is surprisingly undetectable. When the

30   cells are co-transfected with HCMV-RV_La425-kappa/HCMV-CV_H425-

gamma-1, human IgG is produced and it binds to EGFR. How-
ever, when cells are co-transfected with HCMV-CV$_L$425-kappa/
HCMV-RV$_H$a425-gamma-1, human IgG is produced but it does not
bind to EGFR at detectable levels. From these unexpectable
5 results, it is clear that further inventive modifications in
the FRs of reshaped human 425 V$_H$ are necessary in order to get
a functional antigen-binding site.

Modifications in the FRs of reshaped human 425 V$_H$ region:
10

Further changes in the FRs of reshaped human 425 V$_H$ region are
made based on a molecular model of the mouse 425 variable
region domains. The CDR loops of the reshaped human V$_H$ region
are examined to see how they fit into the canonical struc-
15 tures described by Chothia et al., 1989. As a result of this
analysis, certain changes in the FRs are made. Other changes
in the FRs are made based on a functional reshaped human
anti-Tac antibody that was also designed based on human FRs
from subgroup I (Queen et al., 1989). Surprisingly, the V$_H$
20 region of mouse anti-Tac antibody is approximately 79 %
identical to the V$_H$ region of mouse 425 antibody. Now, accord-
ing to the invention, a molecular model of the mouse 425
variable regions is made (Figure 5). The model is based on
25 the structure of HyHEL-5, a structurally-solved antibody
whose variable regions exhibit a high degree of homology to
those of mouse 425 antibody. As a result of the above analy-
sis, amino acid residues at positions 30, 48, 67, 68 and 71

30

in the reshaped human 425 V_H region are changed to be identical to the amino acids occurring at those positions in mouse 425 V_H region. To dissect the individual effects of these changes, a variety of combinations of these changes are constructed and tested according to the invention.

In total, 8 new versions of the reshaped human 425 V_H region are constructed (see Figure 4). From the versions generated by the methods described in detail in the examples, other versions are made by recombining small DNA fragments from previous versions. Once all the desired versions are assembled preferably in pUC18, the reshaped human 425 V_H regions are transferred as HindIII-BamHI fragments into the HCMV-V_H expression vector thus generating versions "b" to "i" of plasmid HCMV-RV$_H$425-gamma-1 (Figure 4).

Modifications in the FRs of reshaped human 425 V_L region:

Although the corresponding cells co-transfected with vectors expressing the reshaped human 425 light chain, version "a", and chimeric 425 heavy chain do produce an antibody that bound to EGFR, the antibody with the reshaped human 425 light chain does not appear to bind as well as chimeric 425 antibody. Examination of the V_L regions of mouse 425 and reshaped human 425 version "a" reveal that residue 71, which is part of the canonical structure for CDR-1 (L1), is not retained in version "a" (Chothia et al., 1989). The PCR-mutagenesis

method (Kamman et al., 1989) is preferably used to introduce a Phe to Tyr change at this position. The HindIII-BamHI fragment generated from this mutagenesis is introduced into the HCMV-V_L expression vector to generate HCMV-RV_Lb425-kappa (Figure 4).

Analysis of the new versions of reshaped human 425 V_H region:

The expression vectors containing reshaped human V_H versions "a" to "i" are co-transfected into the above characterized cells with the expression vector containing reshaped human V_L region version "a". After about 3 days, the cell supernatants are analyzed by ELISA for human IgG production. Levels of production vary between 50-500 ng/ml. The samples are then analyzed by ELISA for human IgG capable fo binding to EGFR. The different versions of reshaped human VH regions result in a wide variety of levels of antigen binding (Figure 6). In this ELISA assay for antigen binding, the various reshaped human 425 antibodies can be directly compared with chimeric 425 antibody, but no to mouse 425 antibody. This is because the antibody used to detect binding to antigen is an anti-human IgG antibody. The nine versions of reshaped human V_H region can be grouped according to their ability to bind to EGFR. Reshaped human V_H region version "g" and "i" provide the highest levels of binding, followed by version "c", "f", and "h", and then followed by version "b". In some experiments, version "e" gives low, but detectable, levels of binding. Versions "a" and "d" never give detectable levels of binding.

A competition binding assay is used to directly compare the
reshaped human 425 antibodies containing versions "g" and "i"
of V_H, and the chimeric 425 antibody, to mouse 425 antibody
(Figure 7). Since the antibodies in the cell supernatants are
5   not purified and are, therefore, quantitated by ELISA, the
results from the competition-binding assay are regarded as
giving relative levels of binding rather than an accurate
quantitation of affinity. Competition binding assays with
samples from four experiments in, for example, COS cells
10   provide consistent results with respect to relative levels of
binding to antigen. Chimeric 425 antibody compete well with
the labelled mouse 425 antibody and give a percent inhibition
of binding just slightly less than that obtained when unla-
belled mouse 425 antibody is competed with labelled mouse 425
15   antibody (Figure 7, Panel A). Reshaped human antibody with V_La
and V_Hg is better than that with V_La and V_Hi region (Figure 7,
Panel B). Comparison of the plateau points of the binding
curves indicates that the reshaped human antibody with V_Hg
competes with labelled mouse 425 antibody 60-80 % as well as
20   the unlabelled mouse 425 antibody does in the same assay.
When the results using samples from four independent experi-
ments in, for example, COS or CHO cells were averaged,
reshaped human antibody containing V_La and V_Hg give a binding
that is 60-80 % that of mouse 425 antibody.
25

Based on these results, it is possible to comment on the
relative contributions of individual residues in the FRs make
to antigen binding. The most significant single change in
this study is the L71V change. Without this change, surpris-
30   ingly, no binding to antigen is detectable (compare versions

"a" and "b" of V_H). The R67K and V68A changes are, surprisingly, also important for binding (compare versions "b" and "c", and versions "i" and "h" of V_H). While introduction of V48KI change alone, and V48I and S30T together, fail to produce significant antigen binding, changes at these positions do enhance antigen binding. The S30T change, surprisingly seems to have a greater effect than the V48I change (compare versions "g" and "i", and versions "f" and "i" of V_H).

Analysis of the new version of reshaped human 425 V_L region:

The expression vector containing the $RV_L b425$ was co-transfected into appropriate preferably eukaryotic cells with the expression vector containing reshaped human V_H region versions "b", "c" or "g". Cell supernatants are harvested and assayed for human IgG production and then for human IgG capable of binding to EGFR (Figure 8, Panel A). These results show that version "b" of reshaped human 425 V_L region increases the binding to antigen. A competition binding assay is then carried out to compare reshaped human 425 antibodies with $V_L a$ plus $V_H g$ and $V_L b$ plus $V_H g$ to mouse 425 antibody. Reshaped human MAb 425 with version "b" of the V_L region has a greater avidity for antigen. Thus, a F71Y change in the V_L increases antigen binding. The reshaped human MAb 425 with $V_L b$ and $V_H g$ has an avidity for antigen 60-80 % of that of the murine MAb 425.

From other experiments, using a reshaped human antibody containing V_Lb plus V_Hg (Examples 10, 11) it can be seen, that the binding potency to EGFR is similar for chimeric, reshaped and murine antibodies.

The invention demonstrates that relatively conservative changes in the FR residues can strongly influence antigen-binding.

The molecular model of mouse 425 variable regions clearly shows this residue at position 30 in V_H to be on the surface of the molecule, in the vicinity of CDR-1. In fact, H1, as defined by Chothia and Lesk, 1987, extends from residues 26 to 32, thus encompassing the residue at position 30. When the residue at position 30 is changed from Ser to Thr in the CAMPATH-1H antibody, it has no effect on antigen binding. When position 30 is changed from Ser to Thr in reshaped human V_H425, binding to antigen is improved. It appears that the amino acid at position 30 does play a role in antigen binding in this particular antibody-antigen interaction. Since the S30T change only improves antigen binding slightly and since the change is not essential for antigen binding, the Thr at position 30 has only a weak interaction with the antigen.

The residue change at position 71 in V_H strongly influences antigen binding. This is surprising since the two residues tested at this position, Val and Leu, only differ by one methyl group. H2 of mouse 425 antibody is a member of H2, group 2 canonical structures as defined by Chothia et al., 1989. HyHEL-5 has an H2 with an amino acid sequence similar to that of the H2 of mouse 425 antibody. In HyHEL-5, a Pro at

position 52A in CDR-2 packs into a cavity created by the
small amino acid (Ala) at position 71 in the FRs. In the
model of the mouse 425 variable regions, there is a similar
interaction between Pro-52A and Val-71. Although in mouse 425

5   V_H the Pro at position 52A is able to pack into the cavity
created by Val at position 71, replacement of Val-71 with a
Leu causes molecular clashing that could alter the conforma-
tion of the CDR-2 loop. For this reason, the V71L change in
reshaped human VH425 re-creates the CDR-2-FR interaction as

10   it occurs in mouse 425 V_H. This, surprisingly, greatly
improves the antigen-binding properties of the reshaped human
425 antibodies (compare reshaped human antibodies with ver-
sions "a" and "b" of V_H in Figure 6).

15   The change at position 71 in V_L probably affects CDR conforma-
tion because residue 71 is a member of the proposed canonical
structure for L1 (CDR-1) (Chothia et al., 1989). Residue 29
in CDR-1 is a buried residue and has a contact with residue
71 in the FRs. In mouse 425 antibody, residue 71 in V_L is Tyr.

20   In the human FRs used for constructing the reshaped human V_Ls,
it is a Phe. It appears that the hydroxyl group found in Tyr,
but not in Phe, has a role in maintaining the correct confor-
mation of CDR-1.

25   From the molecular model of the mouse 425 variable regions,
it appears that Lys-66 forms a salt bridge with Asp-86.
Introduction of larger Arg residue at position 66 would
disrupt the structure. Ala-67 may interact with CDR-2 and
simultaneously changing residues 66 and 67 to Arg and Val, as

30   in V_Ha425, could have an adverse steric effect on CDR-2. The

residue at position 48 is known to be buried (Chothia and
Lesk, 1987), and the model confirms this. Changing residue 48
from an Ile, as found in mouse 425 antibody, to a Val, as
found in human V_H regions of subgroup I, could affect antigen
5   binding by generally disrupting the structure. The amino acid
at position 48 is also close to CDR-2 and may have a subtle
steric effect on the CDR-2 loop.

From the competition binding studies, the best reshaped human
10   V_L and V_H regions are $V_L b$ and $V_H g$. $V_H g$ has all 5 of the FR
changes discussed above plus the change at position 94 that
is included in the first version of reshaped human 425 V_H
region. The FRs in version "b" of reshaped human 425 V_L region
are 70 % identical to those in mouse 425 V_L region. The FRs in
15   version "g" of reshaped human 425 V_H region are 80 % identical
to those in mouse.

<u>Therapeutic and diagnostic use of the antibodies:</u>

20

The antibodies according to the invention can be administered
to human patients for therapy or diagnosis according to known
procedures. Typically the antibody, or antibody fragments,
will be injected parenterally, preferably intraperitoneally.
However, the monoclonal antibodies of the invention can also
25   be administered intravenously.

Determination of appropriate titers of antibody to administer
is well within the skill of the art. Generally, the dosage
ranges for the administration of the monoclonal antibodies of
30   the invention are those large enough to produce the desired
tumor suppressing effect. The dosage should not be so large

as to cause adverse side effects, such as unwanted cross reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of

5   skill in the art. The dosage can be adjusted by the individual physician in the event of any counter indications, immune tolerance or similar conditions. Dosage can vary from 0.1 mg/kg to 70 mg/kg, preferably 0.1 mg/kg to 500 mg/kg/dose, in one or more doses administrations daily,

10   for one or several days.

Preparations for parenteral administration includes sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, poly-

15   ethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ring-

20   er's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present such as for example,

25   antimicrobials, antioxidants, chelating agents, and inert gases and the like.

The antibodies can be conjugated to a toxin such as ricin subunit A, diptheria toxin, or toxic enzyme. Alternatively it

30   can be radiolabelled according to known methods in the art. However, the antibody of the present invention display excellent cytotoxicity, in the absence of toxin, in the presence of effector cells, i.e. human monocytes.

.Solid tumors which can be detected and treated using the present methods include melanoma, glioma and carcinoma. Cancer cells which do not highly express EGFR-receptors can be induced to do so using lymphokine preparations. Also

5 lymphokine preparations may cause a more homogenous expression of EGF-receptors among cells of a tumor, leading to more effective therapy.

Lymphokine preparations suitable for administration include

10 interferon-gamma, tumor necrosis factor, and combinations thereof. These can be administered intravenously, Suitable dosages of lymphokine are 10,000 to 1,000,000 units/patient.

For diagnostic purposes the antibody can be conjugated to a

15 radio-opaque dye or can be radiolabelled. A preferred labelling method is the Iodogen method (Fraker et al., 1978). Preferably the antibody will be administered as $F(ab')_2$ fragments for diagnostic purposes. This provides superior results so that background substraction is unnecessary.

20 Fragments can be prepared by known methods (e.g., Herlyn et al., 1983). Generally pepsin digestion is performed at acid pH and the fragments are separated from undigested IgG and heavy chain fragments by Protein A-Sepharose™ chromatography.

25 The reshaped human 425 antibodies according to the invention are less likely than either mouse or chimeric 425 antibodies to raise an immune response in humans. The avidity of the best version of reshaped human 425 antibody equals that of mouse or chimeric 425 antibody in the best embodiments of the

30 invention. Binding studies show that the potency to compete with EGF for binding to EGFR under optimized

conditions is the same for chimeric, reshaped and murine antibodies. Moreover, the reshaped human 425 antibodies are more efficacious, when used therapeutically in humans, than either the mouse or chimeric 425 antibodies. Due to the great reduction in immunogenicity, the reshaped human 425 antibody has a longer half-life in humans and is the least likely to raise any adverse immune response in the human patient.

The results of the defined MAb 425 show that humanized monoclonal antibodies having an artificial consensus sequence do not effect a remarkable minimum response. Further advantages are described above in the paragraph: Summary of the Invention.

Therefore, the value of the new antibodies of the invention for therapeutic and diagnostic purposes is extraordinarily high.

References cited in the specification:

Amit et al. (1986), Science 233, 747

Aviv et al. (1972), Proc. Nat. Acad. Sci. USA 69, 1408

Bernstein et al. (1977), J. Mol. Bio. 112, 525

Breathnach et al. (1978), Proc. Natl. Acad. Sci USA 75, 4853

Brooks et al. (1983), J. Comp. Chem 4, 187

Brüggemann et al. (1987), J. Exp. Med. 166, 1351

Carter et al. (1985), Oligonucleotide Sitedirected Mutagenesis in M 13, an Experimental Approach Manual, Anglian Biotechnology Ltd. Colchester

Chirgwin et al. (1979), Biochemistry 18, 5294

Chothia et al. (1987), J. Mol. Biol. 196, 901

Chothia et al. (1989), Nature 342, 877

Co et al. (1991), Proc. Natl. Acad. Sci. USA 88, 2869

Cohen (1982), J. Biol. Chem. 258, 1523

Downward et al. (1984), Nature 307, 521

Epp et al. (1975), Biochemistry 14, 4943

Epp et al. (1983), Eur. J. Biochem. 133, 51

Fraker et al. (1978), Biochem. Biophys. Res. Commun. 80, 849

Gillis et al. (1990), Hum. Antibod. Hybridomas 1, 47

Giorgi et al. (1983), Transplant. Proc. 15, 639

Gorman et al. (1991), Proc. Natl. Acad. Sci. USA 88, 4181

Gubler et al. (1983), Gene 25, 263

Hale et al. (1988), Lancet, ii, 1394

Herlyn et al. (1983), Cancer Res. 43, 2731

Hoggenboom et al. (1990), J. Immunol. 144, 3211

Jaffers et al. (1986), Transplantation 41, 572

Jones et al. (1986), Nature 321, 14

Kaariten et al. (1983), J. Immunol. 130, 937

Kabat et al. (1987), Sequences of Proteins of Immunological Interest. US Dept. Health and Human Services, US Government Printing Offices

Kammann et al. (1989), Nucleic Acids Res. 17, 5404

Koprowski et al. (1985), Somatic Cell and Mol. Genetics 11, 297

Kozak (1987), J. Mol. Bio. 196, 947

Levy et al. (1987), Gene 54, 167

Liu et al. (1987), Proc. Natl. Acad. Sci. USA 84, 3439

LoBuglio et al. (1989), Proc. Natl. Acad. Sci. USA 86, 4220

Maeda et al. (1991), Hum. Antibod. Hybridomas 2, 124

Martin (1990), D. Phil. thesis, Oxfor University

Martin et al. (1989), Proc. Natl. Acad. Sci. USA 86, 9268

Mathieson et al. (1990), N. Eng. J. Med. 323, 250

Murthy et al. (1987), Arch. Biochem. Biophys. 252, 549

Nakamaye et al. (1986), Nucleic Res. 14, 9679

Padlan et al. (1989), Proc. Natl. Acad. Sci. USA 86, 5938

Panka et al. (1988), Proc. Natl. Acad. Sci. USA 85, 3080

Queen et al. (1989), Proc. Natl. Acad. Sci. USA 86, 10029

Rabbitts et al. (1984), Curr. Top. Microbiol. Immunol. 113,
5   166

Rechavi et al. (1983), Proc. Natl. Acad. Sci. USA 80, 855

Reichmann et al. (1988), Nature 322, 21

Rodeck et al. (1987), Cancer Res. 47, 3692

Sayers et al. (1988), Nucleic Acid Res. 16, 791

10   Schreiber (1983), J. Biol. Chem. 258, 846

Sheriff et al. (1987), Proc. Natl. Acad. Sci. USA 84, 8075

Show et al. (1986), Proteins 1, 267

Suh et al. (1986), Proteins 1, 74

Sun et al. (1987), Proc. Natl. Acad. Sci. USA 84, 214

15   Sutcliffe (1988), Ph. D. thesis, London University

Takahashi et al. (1987), Cancer Res. 47, 3847

Takahashi et al. (1982), Cell 29, 671

Taylor et al. (1985a), Nucleic Acids Res. 13, 8749

Taylor et al. (1985b), Nucleic Acids Res. 13, 8764

20   Tempest et al. (1991), Biol. Technology 9, 266

Ulrich et al. (1984), Nature 309, 418

Verhoeyen et al. (1988), Science 239, 18

Verhoeyen et al. (1991), In Epenetos, A.A. (ed.), Monoclonal
Antibodies: Applications in Clinical Oncology, Chapman and
25   Hall, London, pp. 37

Ward et al. (1989), Nature 341, 544

Whittle et al. (1987), Protein Eng. 1, 499

Williams et al. (1990), Tibtech 8, 256

30

Example 1

Molecular cloning sequencing:

5   Total RNA was isolated from cell line W425-15 (ACCT HB 9629)
        which produces MAb 425. Approximately 9.6×10^7 cells were
        used to produce total RNA using the guanidinium-CsCl method
        (Chirgwin et al., 1979). Supernatants from the cells used for
        total RNA isolation were assayed by ELISA to ensure that the
10   cells were producing the correct MAb in high amounts. Poly(A+)
        RNA was prepared (Aviv and Leder, 1972). Double-stranded cDNA
        was synthesized essentially according to the methods of
        Gubler and Hoffman (1983) except that primers homologous to
        the 5'-regions of the mouse kappa and gamma-2a immunoglobulin
15   constant regions were used to prime first-strand synthesis
        (Levy et al., 1987). The design of the light chain primer was
        a 26-mer (oligonucleotide 1, Table I) which was designed
        based on published data (Levy et al., 1987; Kaariten et al.,
        1983). The design of the heavy chain primer was a 25-mer
20   (oligonucleotide 2, Table I) and designed based on published
        data (Kaariten et al., 1983; Kabat et al., 1987). Primers
        were designed and synthesized on an Applied Biosystems 380B
        DNA Synthesizer and purified on urea-acrylamide gels. After
        second-strand synthesis, the blunt-ended cDNAs were cloned
25   into SmaI-digested pUC18 (commercially available) and trans-
        formed into competent E. coli cells, e.g. DH5-alpha (commer-
        cially available). Colonies were gridded onto agar plates and
        screened by hybridization using ^{32}P-labelled first-strand
        synthesis primers (Carter et al., 1985). Sequencing of dou-
30   ble-stranded plasmid DNA was carried out using Sequenase
        (United States Biochemical Corporation).

Example 2

Construction of chimeric genes:

5   For each variable region, a front 5' and back 3' polymerase
chain reaction (PCR) primer was synthesized (oligonucleotides
3-6, Table I). PCR reactions were set up using 1 ng of pUC18
plasmid DNA containing the cloned cDNA, front and back PCR
primers at a final concentration of 1 µM each, 200 µM of each

10   dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl, and
0.01 % gelatin (w/v). Amplitaq DNA polymerase (Perkin Elmer
Cetus) was added at 2.5 units per assay. After an initial
melt at 94 °C for 1.5 min, 25 cycles of amplification were
performed at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for

15   3 min. A final extension step at 72 °C was carried out for
10 min. PCR reactions were phenol/chloroform extracted twice
and ethanol precipitated before digesting with HindIII and
BamHI. The PCR fragment coding for the V_L or V_H region was
then cloned into an expression vector. This vector contains

20   the HCMV (human cytomelovirus) enhancer and promoter, the
bacterial neo gene, and the SV40 origin of replication. A
2.0 Kb BamHI fragment of genomic DNA coding for the human
gamma-1 constant region (Takahashi et al., 1982) was inserted
in the correct orientation downstream of the V_H region frag-

25   ment (see HCMV-CV_H425-gamma-1 in Figure 1). This vector was
later adapted by removing the BamHI site at the 3'-end of the
constant region fragment thus allowing variable regions to be
directly inserted into the heavy chain expression vector as
HindIII-BamHI fragments (Maeda et al., 1991). The fragment

30   coding for the V_L region was inserted into a similar HCMV

expression vector   in this case containing a BamHI fragment
of genomic DNA, approximately 2.6 Kb in size, coding for the
human kappa constant region and containing a splice acceptor
site and a poly(A$^+$) (Rabbitts et al., 1984) (see HCMV-CV$_L$-425-
5   kappa in Figure 1).

Example 3

Molecular modelling of MAb 425 V$_L$ and V$_H$:

10

A molecular model of the variable regions of murine MAb 425
was built on the solved structure of the highly homologous
anti-lysozyme antibody, HyHEL-5 (Sheriff et al., 1987). The
variable regions of MAb 425 and HyHEL-5 have about 90 %
15   homology.

The model was built on a Silicon Graphics Iris 4D workstation
running UNIX and using the molecular modeling package
"QUANTA" (Polygen Corp.). Identical residues in the framework
20   were retained; non-identical residues were substituted using
the maximum overlap (Snow and Amzel, 1986) incorporated into
QUANTA's protein modeling facility. The main chain conforma-
tion of the three N-terminal residues in the heavy chain were
substituted from a homologous antibody structure (HyHEL-10
25   (Padlan et al., 1989)) since their temperature factors were
abnormally high (greater than the mean plus three standard
deviations from the backbone temperature factors) and since
they influence the packing of V$_H$ CDR-3 (H3) (Martin, 1990).

30

The CDR-1 (L1) and CDR-2 (L2) sequences of the V_L region and
the CDR-1 (H1) and CDR-2 (H2) sequences of the V_H region from
MAb 425 corresponded to canonical forms postulated by Chothia
et al. (1989). The main chain torsion angles of these loops

5   were kept as in HyHEL-5. The CDR-3 (L3) sequence of the V_L
region and the CDR-3 (H3) of the V_H region from MAb 425 did
not correspond to canonical structures and, therefore, were
modeled in a different way. The computer program of Martin et
al. (1989) was used to extract loops from the Brookhaven

10 Databank (Bernstein et al., 1977). The loops were then sorted
based on sequence similarity, energy, and structure-determin-
ing residues (Sutcliffe, 1988). The top-ranked loops were
inspected on the graphics and the best selected by eye. H3
was modeled on bovine glutathione peroxidase (Epp et al.,

15 1983) in the region of residues 92-103. L3 was modelled on
the murine IgA (J539) Fab fragment (Suh et al., 1986) in the
region of residues 88-96 of the light chain.

The model was subjected to steepest descents and conjugate

20 gradients energy minimization using the CHARm potential
(Brooks et al., 1983) as implemented in QUANTA in order to
relieve unfavorable atomic contacts and to optimize Van der
Waals and electrostatic interactions.

25

30

- 54 -

Example 4

Construction of humanized antibody genes:

5   The construction of the first version of the reshaped human
        425 light chain was carried out using a CDR-grafting approach
        similar to that described by Reichmann et al. (1988) and
        Verhoeyen et al. (1988). Single-stranded template DNA was
        prepared from a M13mp18 vector (commercially available)
10   containing a HindIII-BamHI fragment coding for the human
        anti-lysozyme V_L region (EP 239 400, G. Winter). The FRs of
        this light chain are derived from the crystallographically-
        solved protein REI. Three oligonucleotides were designed
        which consisted of DNA sequences coding for each of the mouse
15   MAb 425 light chain CDRs flanked on each end by 12 bases of
        DNA complementary to the DNA sequences coding for the adja-
        cent FRs of human REI (oligonucleotides 7-9 in Table I).
        Oligonucleotides were synthesized and purified as before. All
        three oligonucleotides were phosphorylated and used simulta-
20   neously in an oligonucleotide-directed in vitro mutagenesis
        system based on the methods of Eckstein and coworkers (Taylor
        et al., 1985; Nakamaye and Eckstein, 1986; and Sayers et al.,
        1988). The manufacturer's instructions were followed through
        the exonuclease III digestion step. The reaction was then
25   phenol/chloroform extracted, ethanol precipitated, and resus-
        pended in 100 µl of TE. A volume of 10 µl was used as tem-
        plate DNA in a 100 µl PCR amplification reaction containing
        M13 universal primer and reverse sequencing primer to a final
        concentration of 0.2 µM each. Buffer and thermocycling condi-
30   tions were as described in Example 2 with the exception of
        using a 55 °C annealing temperature. The PCR reaction was
        phenol/chloroform extracted twice and ethanol precipitated

before digestion with HindIII and BamHI and subcloning into
pUC18. Putative positive clones were identified by hybridiza-
tion to ^{32}P-labelled mutagenic primers (Carter et al., 1987).
Clones were confirmed as positive by sequencing. A V_L region

5   containing all three grafted CDRs was cloned as a HindIII-
BamHI fragment into the V_L expression vector to create the
plasmid HCMV-RV$_L$a425-kappa.

Version "b" of the reshaped V_L was constructed using the PCR

10   mutagenesis method of Kammann et al. (1989), with minor
modifications. The template DNA was the RV$_L$a subcloned into
pUC18. The first PCR reaction was set up in a total volume of
50 μl and contained 1 ng template, M13 reverse sequencing
primer and primer 10 (Table I) at a final concentrations of 1

15   μM, 200 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM
MgCl, and 0.01 % (w/v) gelatin. Amplitaq DNA polymerase was
added at a concentration of 1 unit per assay. The reaction
was set up in triplicate. After melting at 94 °C for 1.5 min,
the reactions were cycled at 1 min 94 °C, 1 min 37 °C, and 2

20   min 72 °C for 40 cycles, followed by an extension at 72 °C for
10 min. The reactions were pooled, phenol/chloroform
extracted and ethanol precipitated before isolating the PCR
product from a TAE agarose gel. A tenth of the first PCR
reaction was then used as one of the primers in the second

25   PCR reaction. The second reaction was as the first except the
first reaction product and 20 pmol of M13 universal primer
were used. Cycling was as described by Kammann et al. (1989).
The HindIII-BamHI fragment was cloned into pUC18 and
sequenced. A DNA fragment bearing the desired change was

30   subcloned into the V_L expression plasmid to create plasmid
HCMV-RV$_L$b425-kappa.

The first version of the reshaped human V_H region of 425 was
chemically synthesized. A DNA sequence was designed coding
for the required amino acid sequence and containing the
necessary flanking DNA sequences (see above). Codon usage was
5   optimized for mammalian cells with useful restriction enzyme
sites engineered into the DNA sequences coding for FRs. The
454 bp was synthesized and subcloned into pUC18 as an EcoRI-
HindIII fragment. A HindIII-BamHI fragment coding for the
reshaped humanized 425 heavy chain was then transferred into
10   the V_H expression vector, to produce the plasmid HCMV-
RV_Ha-425-gamma-1.

Eight other versions of the reshaped humanized heavy chains
were constructed by a variety of methods. The HindIII-BamHI
15   fragment coding for the version "a" of the heavy chain was
transferred to M13mp18 and single-stranded DNA prepared.
Using oligonucleotides 11-13 (Table I), PCR-adapted M13
mutagenesis, as described above, was used to generate DNA
coding for reshaped human 425 V_H regions versions "d", "e",
20   "f" and "g" in pUC18. These versions were subcloned into the
heavy chain expression vector as HindIII-BamHI fragments to
create plasmids HCMV-RV_Hd425-gamma-1, HCMV-RV_He425-gamma-1,
HCMV-RV_Hf425-gamma-1, and HCMV-RV_Hg425-gamma-1.

25

Reshaped human 425 V_H regions versions "b" and "c" were
generated using the PCR mutagenesis method of Kammann et al.
(1989) as described above. The template DNA was reshaped
human 425 V_H region version "a" subcloned into pUC18, and the
30   mutagenic primer used in the first PCR reaction was either

primer 13 or 14 (Table I). After mutagenesis and sequencing,
sequences bearing the desired changes were subcloned into the
heavy chain expression plasmid to create plasmids. HCMV-
RV$_H$b425-gamma-1 and HCMV-RV$_H$c425-gamma-1.

5

Reshaped heavy chain versions "h" and "i" were constructed
from the pUC-based clones of existing versions. A 0.2 Kb
HindIII-XhoI fragment from version "e" was ligated to a
2.8 Kb XhoI-HindIII fragment from either version "b" or "c"

10   producing the new versions "h" and "i", respectively, The
HindIII-BamHI fragments coding for these versions were sub-
cloned into the heavy chain expression vector to produce the
HCMV-RV$_H$h425-gamma-1 and HCMV-RV$_H$i425-gamma-1.

15   **Example 5**

Transfection of DNA into COS cells:

COS cells were electroporated with 10 µg each of the expres-
20   sion vectors bearing the genes coding for the heavy and light
chains. Briefly, 10 µg of each plasmid was added to a 0.8 ml
aliquot of a 1 x 10^7 cells/ml suspension of COS cells in PBS.
A Bio-Rad™ Gene Pulser was used to deliver a pulse of 1900 V,
with a capacitance of 25 µF. The cells were left to recover
25   at room temperature for 10 min before plating into 8 ml DMEM
containing 10 % fetal calf serum. After 72 h incubation, the
media was collected, centrifuged to remove cellular debris,
and stored under sterile conditions at 4 °C for short periods,
or at -20 °C for longer periods, prior to analysis by ELISA.

30

Example 6

The transfection of DNA into CHO cells was done according to Example 5.

5

Example 7

Quantification of IgG production and detection of antigen binding:

10

Human IgG present in COS cell supernatants was detected by ELISA: In the ELISA assay for human IgG, 96-well plates were coated with goat anti-human IgG (whole molecule) and human IgG in the samples that bound to the plates was detected
15   using alkaline phosphatase-conjugated goat anti-human IgG (gamma-chain specific). Purchasable purified human IgG was used as a standard. Binding to the antigen recognized by MAb 425 was determined in a second ELISA. Plates were coated with an EGFR protein preparation (obtainable, for example, accord-
20   ing to Rodeck et al., 1980) and antibodies binding to EGFR were detected using either an anti-human IgG (gamma-chain specific) peroxidase conjugate (for chimeric and reshaped human antibodies) or an anti-mouse IgG (whole molecule) peroxidase conjugate (for the mouse MAb 425 antibody) (both
25   conjugates supplied by Sigma). Purified murine MAb 425 was used as a standard.

30

Example 8

Competition binding assay:

5   Murine MAb 425 was biotinylated using a correspondingly
       purchasable kit. ELISA plates were coated with an optimal
       dilution of the EGFR protein. Dilutions of the COS cell
       supernatants, in a volume of 50 µl, were mixed with 50 µl of
       the biotinylated murine MAb 425 (estimated by ELISA to be
10   1.75 µg/ml). Each COS cell supernatant was tested in dupli-
       cate. Plates were incubated at room temperature, overnight.
       Bound biotinylated murine MAb 425 was detected by the addi-
       tion of a purchasable streptavidin horseradish peroxidase
       complex. A control with no competitor present allowed a value
15   of percentage of inhibition or blocking to be calculated for
       each COS cell supernatant as follows:

$$100 - [(OD_{450} \text{ of sample } / OD_{450} \text{ of control}) \times 100]$$

20   **Example 9**

       Different probes of murine, reshaped and chimeric MAb 425
       were analyzed by SDS-Polyacrylamide-Gelspaceelectrophoresis
       (SDS-PAGE) according to Laemmli et al. 2.5 µg of each sample
25   were applied to each well under non-reducing as well as under
       reducing conditions. Protein was visualized by Coomassie
       staining. Fig. 9 (A) shows that the samples have similar
       purity.

30   MW range of the antibodies: 180,000 - 200,000.

- 60 -

Example 10

Reshaped MAb 425 was purified by gelspacefiltration on Super-
ose 12™ (Pharmacia Corp. Sweden) according to standard meth-
5   ods. The antibody was eluted with PBS (pH 7.4, 0.8 M NaCl)
(0.1 M). A single peak (at 5 min) can be obtained (Fig. 9
(B)).

Example 11

10

Biotin-labelled MAb 425 was used to compete with unlabelled
MAb 425 or derivates for binding to EGFR. Biotin-labelling
occurred according to standard methods. EGFR was solubilized
from A431 membranes by standard methods. A431 cells were
15   commercially purchased. Detection was done after incubation
with POD-conjugated streptavidin and substrate. From this
data inhibition curves were constructed (Fig. 10). The curves
show that the binding of the various antibodies are compara-
ble.

20

Example 12

Different probes of purified murine, chimeric and reshaped
MAbs 425 were tested for their potency to compete with EGF
25   regarding their binding to EGFR. The test was performed by
competing ^{125}I-labelled EGF (Amersham Corp., GB) and various
antibodies for binding to EGF-receptor positive membranes
(A431). The test system is based on SPA technology (Amer-
sham). The competition curves of the murine and the reshaped
30   antibodies (3 probes) are nearly identical (Fig. 11).

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Patent Claims

1. Humanized monoclonal antibody comprising antigen binding sites (CDRs) of non-human origin, and the FRs of variable region and constant regions of light and heavy chains of human origin, characterized in that at least the FRs of the variable region of the heavy chain comprise a modified consensus sequence of different variable regions of a distinct class or subgroup of a human immunoglobulin.

2. Humanized monoclonal antibody according to Claim 1, wherein the FRs of the consensus sequence has a homology of at least 70 % compared with the amino acid sequence of the FRs of the variable regions of the non-human antibody from which the antigen binding sites originate.

3. Humanized monoclonal antibody according to Claim 1 or 2, having the following properties:

   (a) binds to human EGF-receptors;

   (b) inhibits binding of EGF to EGF-receptor;

   (c) inhibits the EGF-dependent tyrosine kinase activity of EGF-receptor;

   (d) inhibits the growth of EGF-sensitive cells.

4. Humanized monoclonal antibody according to Claim 3, wherein the hypervariable regions of the antigen binding sites comprise the following amino acid sequences:

5   **light chain**

   CDR-1   -Ser-Ala-Ser-Ser-Ser-Val-Thr-Tyr-Met-Tyr-
   CDR-2   -Asp-Thr-Ser-Asn-Leu-Ala-Ser-
   CDR-3   -Gln-Gln-Trp-Ser-Ser-His-Ile-Phe-Thr-

10   **heavy chain**

   CDR-1   -Ser-His-Trp-Met-His-
   CDR-2   -Glu-Phe-Asn-Pro-Ser-Asn-Gly-Arg-Thr-Asn-Tyr-Asn-
15   Glu-Lys-Phe-Lys-Ser-
   CDR-3   -Arg-Asp-Tyr-Asp-Tyr-Asp-Gly-Arg-Tyr-Phe-Asp-Tyr-

5. Humanized monoclonal antibody according to Claim 4, wherein the FRs of the variable region which is not
20   related to the antigen binding sites comprise the follow- ing amino acid sequence:

     light chain

25   FR-1   -Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-
             Ala-Ser-Val-Gly-Asp-Arg-Val-Thr-Ile-Thr-Cys-
     FR-2   -Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Lys-Ala-Pro-Lys-Leu-
             Leu-Ile-Tyr-

30

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```
     FR-3       -Gly-Val-Pro-Ser-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-
                Thr-Asp-Tyr(Phe,Trp,His)-Thr-Phe-Thr-Ile-Ser-Ser-
                Leu-Gln-Pro-Glu-Asp-Ile-Ala-Thr-Tyr-Tyr-Cys-
     FR-4       -Phe-Gly-Gln-Gly-Thr-Lys-Val-Glu-Ile-Lys-
```

5

heavy chain

```
     FR-1       -Gln-Val-Gln-Leu-Val-Gln-Ser-Gly-Ala-Glu-Val-Lys-
                Lys-Pro-Gly-Ala-Ser-Val-Lys-Val-Ser-Cys-Lys-Ala-
10               Ser-Gly-Tyr-Thr-Phe-Thr(Ser)-
     FR-2       -Trp-Val-Arg(His)-Gln-Ala(Lys,His)-Pro(Val)-Gly-
                Gln-Gly-Leu-Glu-Trp-Ile(Val,Leu)-Gly-
     FR-3       -Lys(Arg,His)-Ala(Val-Pro-Gly)-Thr-Met-Thr-
                Val(Ala,Pro,Gly)-Asp-Thr-Ser-Thr-Asn-Thr-Ala-Tyr-
15              Met-Glu(Asn)-Leu-Ser-Ser-Leu-Arg-Ser-Glu-Asp-Thr-
                Ala-Val-Tyr-Tyr-Cys-Ala-Ser-
     FR-4       -Trp-Gly-Gln-Gly-Thr-Leu-Val-Thr-Val-Ser-Ser-,
```

and wherein the amino acids listed in the brackets are
20   alternatives.

     6.   Humanized monoclonal antibody according to Claim 4 or 5,
          wherein the constant regions of the heavy chain comprise
          the amino acid sequence of a gamma-1 chain, and the
25   constant regions of the light chain comprise the amino
          acid sequence of a kappa chain of a human immunoglobulin.

     7.   Humanized monoclonal antibody according to one of the
          Claims 3 to 6, comprising a derivate of an amino acid
30   sequence modified by amino acid deletion, substitution,
          addition or inversion within the variable and constant
          regions wherein the biological function of specific
          binding to the antigen is preserved.

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8. Expression vec⁻or, suitable for transformation of host cells, characterized in that it comprises a DNA sequence coding for the variable and/or constant regions of the light and/or heavy chains of a humanized antibody according to Claims 1 to 7.

5

9. Expression vector according to Claim 8, wherein the DNA sequences are coding for an antibody protein according to one of the antibodies of Claims 3 to 7.

10

10. Expression vector having the designation pRVL425, deposited at DSM under Accession No. DSM 6340.

11. Expression vector having the designation pRVH425, deposited at DSM under Accession No. DSM 6339.

15

12. Humanized or chimeric monoclonal antibody, comprising hypervariable regions (CDRs) of antigen binding sites of murine origin and the FRs of variable regions of human or murine origin and constant regions of light and heavy chains of human origin, characterized in that the hypervariable regions comprise the following amino acid sequences,

20

light chain

25

CDR-1   -Ser-Ala-Ser-Ser-Ser-Val-Thr-Tyr-Met-Tyr-
CDR-2   -Asp-Thr-Ser-Asn-Leu-Ala-Ser-
CDR-3   -Gln-Gln-Trp-Ser-Ser-His-Ile-Phe-Thr-

30

heavy chain

CDR-1	-Ser-His-Trp-Met-His-	
CDR-2	-Glu-Phe-Asn-Pro-Ser-Asn-Gly-Arg-Thr-Asn-Tyr-Asn-	
5		Glu-Lys-Phe-Lys-Ser-
CDR-3	-Arg-Asp-Tyr-Asp-Tyr-Asp-Gly-Arg-Tyr-Phe-Asp-	
	Tyr-,	

and wherein the constant regions of the heavy chain

10 comprise the amino acid sequence of a gamma-1 chain, and

the constant regions of the light chain comprise the

amino acid sequence of a kappa chain of a human immuno-

globulin.

15   13. Humanized monoclonal antibody according to Claim 12,

wherein the FRs of the variable region which is not

related to the antigen binding sites, are of human origin

and comprise the following amino acid sequence,

20 **light chain**

FR-1	-Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-	
	Ala-Ser-Val-Gly-Asp-Arg-Val-Thr-Ile-Thr-Cys-	
FR-2	-Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Lys-Ala-Pro-Lys-Leu-	
25		Leu-Ile-Tyr-
FR-3	Gly-Val-Pro-Ser-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-	
	Thr-Asp-Tyr(Phe,Trp,His)-Thr-Phe-Thr-Ile-Ser-Ser-	
	Leu-Gln-Pro-Glu-Asp-Ile-Ala-Thr-Tyr-Tyr-Cys-	
FR-4	-Phe-Gly-Gln-Gly-Thr-Lys-Val-Glu-Ile-Lys-	

30

66

heavy chain

FR-1   -Gln-Val-Gln-Leu-Val-Gln-Ser-Gly-Ala-Glu-Val-Lys-
        Lys-Pro-Gly-Ala-Ser-Val-Lys-Val-Ser-Cys-Lys-Ala-
5   Ser-Gly-Tyr-Thr-Phe-Thr(Ser)-

FR-2   -Trp-Val-Arg(His)-Gln-Ala(Lys,His)-Pro(Val)-Gly-
        Gln-Gly-Leu-Glu-Trp-Ile(Val,Leu)-Gly-

FR-3   -Lys(Arg,His)-Ala(Val,Pro,Gly)-Thr-Met-Thr-
        Val(Ala,Pro,Gly)-Asp-Thr-Ser-Thr-Asn-Thr-Ala-Tyr-
10   Met-Glu(Asn)-Leu-Ser-Ser-Leu-Arg-Ser-Glu-Asp-Thr-
        Ala-Val-Tyr-Tyr-Cys-Ala-Ser-

FR-4   -Trp-Gly-Gln-Gly-Thr-Leu-Val-Thr-Val-Ser-Ser-

14. Chimeric monoclonal antibody according to Claim 12,
15   wherein the FRs of the variable region which is not
        related to the antigen binding site, are of murine origin
        and comprise the following amino acid sequences:

light chain

20

FR-1   -Gln-Ile-Val-Leu-Thr-Gln-Ser-Pro-Ala-Ile-Met-Ser-
        Ala-Ser-Pro-Gly-Glu-Lys-Val-Thr-Met-Thr-Cys-

FR-2   -Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Ser-Ser-Pro-Arg-Leu-
        Leu-Ile-Tyr-

25 FR-3   -Gly-Val-Pro-Val-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-
        Thr-Ser-Tyr-Ser-Leu-Thr-Ile-Ser-Arg-Met-Glu-Ala-
        Glu-Asp-Ala-Ala-Thr-Tyr-Tyr-Cys-

FR-4   -Phe-Gly-Ser-Gly-Thr-Lys-Leu-Glu-Ile-Lys-

30

heavy chain

FR-1   -Gln-Val-Gln-Leu-Gln-Gln-Pro-Gly-Ala-Glu-Leu-Val-
Lys-Pro-Gly-Ala-Ser-Val-Lys-Leu-Ser-Cys-Lys-Ala-
5   Ser-Gly-Tyr-Thr-Phe-Thr-

FR-2   -Trp-Val-Lys-Gln-Arg-Ala-Gly-Gln-Gly-Leu-Glu-Trp-
Ile-Gly-

FR-3   -Lys-Ala-Thr-Leu-Thr-Val-Asp-Lys-Ser-Ser-Ser-Thr-
Ala-Tyr-Met-Gln-Leu-Ser-Ser-Leu-Thr-Ser-Glu-Asp-
10   Ser-Ala-Val-Tyr-Tyr-Cys-Ala-Ser-

FR-4   -Trp-Gly-Gln-Gly-Thr-Thr-Leu-Thr-Val-Ser-Ser-

15. Expression vector, suitable for transformation of host
cells, characterized in that it comprises DNA sequences
15   coding for the variable and constant regions of the light
and/or heavy chains of a humanized monoclonal antibody
according to Claim 12 or 13, or of a chimeric monoclonal
antibody according to Claim 12 or 14.

20 16. Expression vector having the designation pCVL425, depos-
ited at DSM under Accession No. DSM 6338.

17. Expression vector having the designation pCVH425, depos-
ited at DSM under Accession No. DSM 6337.

25

18. Process for the preparation of a humanized monoclonal
antibody, comprising hypervariable regions (CDRs) of
antigen binding sites of non-human origin, and FRs of the
variable regions and constant regions of the light and
30   heavy chains of human origin by cultivating transformed
host cells in a culture medium and purification and
isolation the expressed antibody proteins, characterized
in

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(a) synthesizing or partially synthesizing or isolating
     an oligonucleotide sequence which codes for an amino
     acid consensus sequence of different FRs of the vari-
     able regions (FR-1 to FR-4) of a heavy chain of a
     class or a subgroup of a human immunoglobulin,
     wherein the used consensus sequence has a homology of
     at least 70 % compared with the amino acid sequence
     of the FRs of the variable regions of the non-human
     antibody from which the antigen binding sites origi-
     nate, and wherein the consensus sequence is modified
     by alterations of maximum 10 % of the amino acids in
     order to preserve the binding capability of the anti-
     gen to the hypervariable regions;

(b) synthesizing or partially synthesizing or isolating
     an oligonucleotide sequence which codes for an amino
     acid consensus sequence under the conditions given in
     (a) of different FRs of the variable regions (FR-1 to
     FR-4) of a light chain of a class or a subgroup of a
     human immunoglobulin, or, alternatively, which codes
     for a corresponding natural occurring amino acid
     sequence;

(c) in each case synthesizing or partially synthesizing
     or isolating an oligonucleotide sequence which codes
     for the amino acid sequence of the hypervariable
     regions (CDRs) of the light and heavy chain corre-
     sponding to the hypervariable regions of the basic
     non-human antibody;

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(d) in each case synthesizing or partially synthesizing or isolating an oligonucleotide sequence which codes for the amino acid sequence of the constant regions of the light and heavy chain of a human immunoglobulin;

(e) constructing one or several expression vectors comprising in each case at least a promoter, a replication origin and the coding DNA sequences according to (a) to (d), wherein the DNA sequences coding for the light and heavy chains can be present together in one or, alternatively, in two or more different vectors,

and finally,

(f) transforming the host cells with one or more of the expression vectors according to (e).

19. Process according to Claim 18, wherein DNA sequences are used coding for the following amino acid sequences which represent the hypervariable regions (CDRs):

light chain

CDR-1   -Ser-Ala-Ser-Ser-Ser-Val-Thr-Tyr-Met-Tyr-
CDR-2   -Asp-Thr-Ser-Asn-Leu-Ala-Ser-
CDR-3   -Gln-Gln-Trp-Ser-Ser-His-Ile-Phe-Thr-

7o

heavy chain

    CDR-1   -Ser-His-Trp-Met-His-

    CDR-2   -Glu-Phe-Asn-Pro-Ser-Asn-Gly-Arg-Thr-Asn-Tyr-Asn-

5   Glu-Lys-Phe-Lys-Ser-

    CDR-3   -Arg-Asp-Tyr-Asp-Tyr-Asp-Gly-Arg-Tyr-Phe-Asp-Tyr-

20. Process according to Claim 18 or 19, wherein DNA
sequences are used coding for the following amino acid

10   sequences which represent the FRs of the variable
regions:

light chain

15   FR-1   -Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-

              Ala-Ser-Val-Gly-Asp-Arg-Val-Thr-Ile-Thr-Cys-

    FR-2   -Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Lys-Ala-Pro-Lys-Leu-

              Leu-Ile-Tyr-

    FR-3   -Gly-Val-Pro-Ser-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-

20   Thr-Asp-Tyr(Phe,Trp,His)-Thr-Phe-Thr-Ile-Ser-Ser-

              Leu-Gln-Pro-Glu-Asp-Ile-Ala-Thr-Tyr-Tyr-Cys-

    FR-4   -Phe-Gly-Gln-Gly-Thr-Lys-Val-Glu-Ile-Lys-

heavy chain

25

    FR-1   -Gln-Val-Gln-Leu-Val-Gln-Ser-Gly-Ala-Glu-Val-Lys-

              Lys-Pro-Gly-Ala-Ser-Val-Lys-Val-Ser-Cys-Lys-Ala-

              Ser-Gly-Tyr-Thr-Phe-Thr(Ser)-

    FR-2   -Trp-Val-Arg-(His)-Gln-Ala(Lys,His)-Pro(Val)-Gly-

30   Gln-Gly-Leu-Glu-Trp-Ile(Val,Leu)-Gly-

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```
FR-3      -Lys(Arg,His)-Ala(Val,Pro,Gly)-Thr-Met-Thr-
          Val(Ala,Pro,Gly)-Asp-Thr-Ser-Thr-Asn-Thr-Ala-Tyr-
          Met-Glu(Asn)-Leu-Ser-Ser-Leu-Arg-Ser-Glu-Asp-Thr-
          Ala-Val-Tyr-Tyr-Cys-Ala-Ser-
FR-4      -Trp-Gly-Gln-Gly-Thr-Leu-Val-Thr-Val-Ser-Ser
```

21. Process for the preparation of a chimeric monoclonal antibody having the biological function of binding to epitopes of the EGF-receptor, comprising hypervariable regions (CDRs) of antigen binding sites and FRs of the variable regions of murine origin   and FRs of the variable regions of murine origin and constant regions of the light and heavy chains of human origin by cultivating transformed host cells in a culture medium and purification and isolation the expressed antibody proteins, characterized in that the host cells are transformed with expression vectors according to one of the expression vectors of Claims 15 to 17.

22. Pharmaceutical composition comprising a humanized monoclonal antibody according to one of the antibodies of Claims 1 to 7 or 12 to 13.

23. Pharmaceutical composition comprising a chimeric monoclonal antibody according to one of the antibodies of Claim 12 or 14.

24. Use of humanized or chimeric antibody according to one of the antibodies of Claims 3 to 7 or 12 to 14 for the manufacture of a drug directed to tumors.

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25. Use of humanized or chimeric antibody according to one of
the antibodies of Claims 3 to 7 or 12 to 14 for diagnos-
tic locating and assessing tumor growth.

5   26. Purified humanized and chimeric monoclonal antibody which
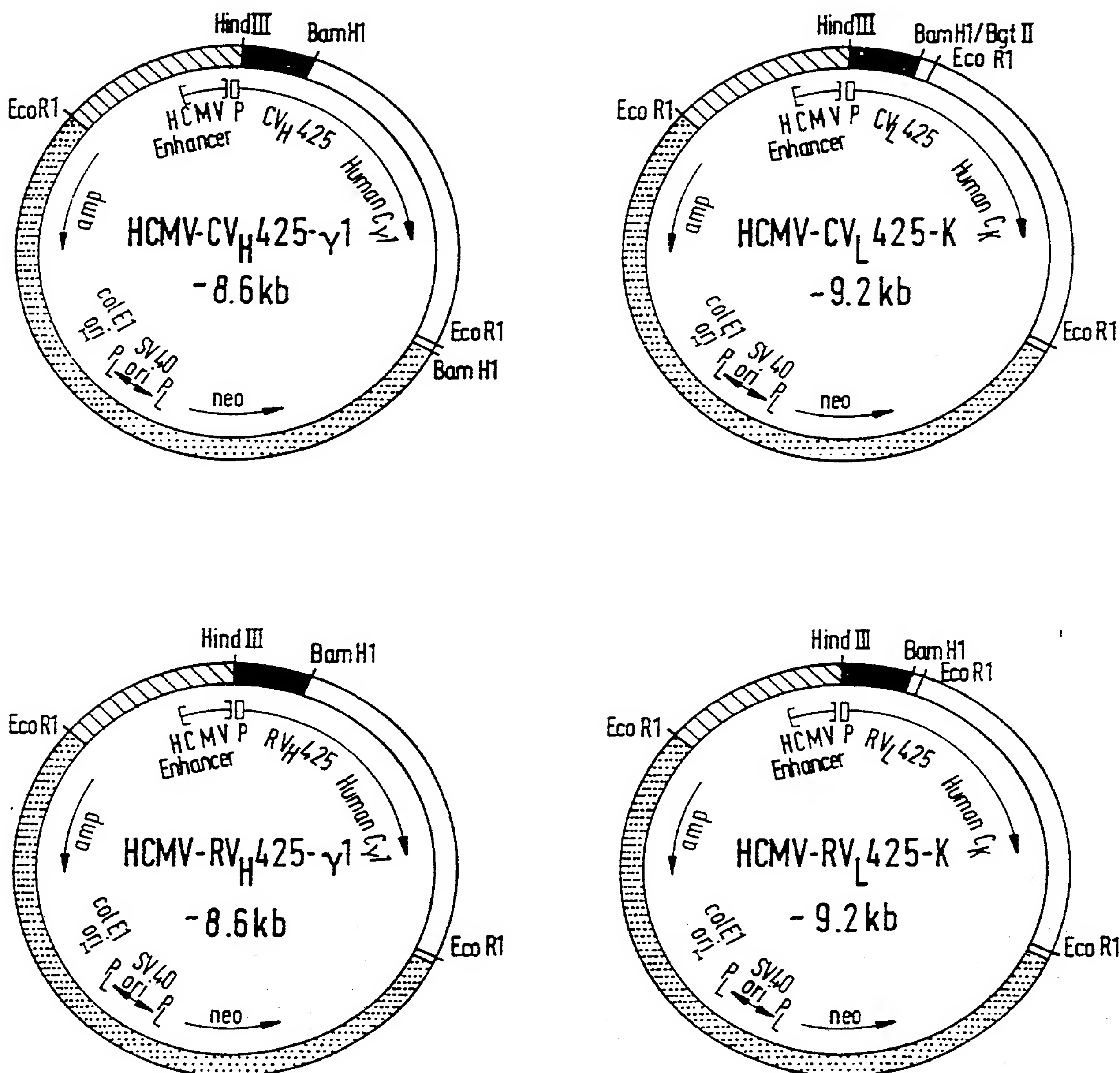derives from murine MAb 425.

10

15

20

25

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FIG.1

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FIG. 2

Panel A:

```
5'-------CGAGCTCGG-CTGAGCACACAGGACCTCACCATG GGT TGG AGC TAT  45
    pUC18----->-3'-GACTCGTGTGTCCTGGAGTGGTACCCA-----5'
              5'-CTCCAAGCTTGACCTCACCATGG-3'
                    ▲
                HindIII                    Met Gly Trp Ser Tyr
```

```
ATC ATC CTC TTT TTG GTA GCA ACA GCT ACA GAT GTC CAC TCC CAG  90
Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Asp Val His Ser Gln
GTC CAG CTG CAA CAA CCT GGG GCT GAA CTG GTG AAG CCT GGG GCT 135
Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala
TCA GTG AAG TTG TCC TGC AAG GCT TCC GGC TAC ACC TTC ACC AGC 180
Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr(Ser
CAC TGG ATG CAC TGG GTG AAG CAG AGG GCT GGA CAA GGC CTT GAG 225
His Trp Met His)Trp Val Lys Gln Arg Ala Gly Gln Gly Leu Glu
TGG ATC GGA GAG TTT AAT CCC AGC AAC GGC CGT ACT AAC TAC AAT 270
Trp Ile Gly(Glu Phe Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn
GAG AAA TTC AAG AGC AAG GCC ACA CTG ACT GTA GAC AAA TCC TCC 315
Glu Lys Phe Lys Ser)Lys Ala Thr Leu Thr Val Asp Lys Ser Ser
AGC ACA GCC TAC ATG CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT 360
Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser
GCG GTC TAT TAC TGT GCC AGT CGG GAC TAT GAT TAC GAC GGA CGG 405
Ala Val Tyr Tyr Cys Ala Ser(Arg Asp Tyr Asp Tyr Asp Gly Arg
```

```
                                      3'-AG TGT CAG AGG AGT
TAC TTT GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA 450
Tyr Phe Asp Tyr)Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
          BamHI
        ▲
CCACTCACCTAGGTT-5'                     <---- pUC15
GCCAAAACAACACCCCATCGGTCTATCCACTGGAT-TCCTCTAGAGTCGACC---3'  501
```

Panel B:

```
    --- pUC18 -------->
5'----TTCGAGCTCGGTACCC-ACAAAATG GAT TTT CAA GTG CAG ATT TTC  45
3'----AAGCTCGAGCCATGGG-TGTTTTAC CTA AAA GTT CAC GTC--5'
        5'-AGAAAGCTT-CCACCATG GAT TTT CAA GTG-3'
              ▲
          HindIII                Met Asp Phe Gln Val Gln Ile Phe
```

```
AGC TTC CTG CTA ATC AGT GCC TCA GTC ATA CTG TCC AGA GGA CAA  90
Ser Phe Leu Leu Ile Ser Ala Ser Val Ile Leu Ser Arg Gly Gln
ATT GTT CTC ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG 135
Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
GAG AAG GTC ACT ATG ACC TGC AGT GCC AGC TCA GTG TAC TAC 180
Glu Lys Val Thr Met Thr Cys(Ser Ala Ser Ser Ser Val Thr Tyr
ATG TAT TGG TAC CAG CAG AAG CCA GGA TCC TCC CCC AGA CTC CTG 225
Met Tyr)Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu
ATT TAT GAC ACA TCC AAC CTG GCT TCT GGA GTC CCT GTT CGT TTC 270
Ile Tyr(Asp Thr Ser Asn Leu Ala Ser)Gly Val Pro Val Arg Phe
AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC CGA 315
Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg
ATG GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG CAG TGG AGT 360
Met Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys(Gln Gln Trp Ser
```

```
                                      3'-C AAC CTT TAT TTT
AGT CAC ATA TTC ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA 405
Ser His Ile Phe Thr)Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
GCACTCATCTAGATG-5'   BglII                    <-- pUC15
                  ▲
CGGGCTGATGCTGCACCAACTGTATGGATCTTCCCACCATCCAGGATCC-GGGGATCC-3'  462
```

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FIG. 3

```
         HindIII
5'--AAGCTTGCCGCCACC ATG GAC TGG ACC TGG CGC GTG TTT TGC CTG   45
                    Met Asp Trp Thr Trp Arg Val Phe Cys Leu

CTC GCC GTG GCT CCT GGG GCC CAC AGC CAG GTG CAA CTA GTG CAG   90
Leu Ala Val Ala Pro Gly Ala His Ser Gln Val Gln Leu Val Gln

TCC GGC GCC GAA GTG AAG AAA CCC GGT GCT TCC GTG AAG GTG AGC   135
Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val Ser

TGT AAA GCT AGC GGT TAT ACC TTC TCT TCC CAC TGG ATG CAT TGG   180
Cys Lys Ala Ser Gly Tyr Thr Phe Ser(Ser His Trp Met His)Trp

GTT AGA CAG GCC CCA GGC CAA GGG CTC GAG TGG GTG GGC GAG TTC   225
Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Val Gly(Glu Phe

AAC CCT TCA AAT GGC CGG ACA AAT TAT AAC GAG AAG TTT AAG AGC   270
Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn Glu Lys Phe Lys Ser)

AGG GTT ACC ATG ACC TTG GAC ACC TCT ACA AAC ACC GCC TAC ATG   315
Arg Val Thr Met Thr Leu Asp Thr Ser Thr Asn Thr Ala Tyr Met

GAA CTG TCC AGC CTG CGC TCC GAG GAC ACT GCA GTC TAC TAC TGC   360
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys

GCC TCA CGG GAT TAC GAT TAC GAT GGC AGA TAC TTC GAC TAT TGG   405
Ala Ser(Arg Asp Tyr Asp Tyr Asp Gly Arg Tyr Phe Asp Tyr)Trp

                                            BamHI   EcoRI
GGA CAG GGT ACC CTT GTC ACC GTC AGT TCA GGTGAGTGGATCCGAATTC   454
Gly Gln Gly Thr Leu Val Thr Val Ser Ser
```

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FIG. 4-1

Panel A:

V_L425	Gln-Ile-Val-Leu-Thr-Gln-Ser-Pro-Ala-Ile-	
RV_L a425	Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-	
RV_L b425	--	
V_L425	Met-Ser-Ala-Ser-Pro-Gly-Glu-Lys-Val-Thr-	FR-1
RV_L a425	Leu-Ser-Ala-Ser-Val-Gly-Asp-Arg-Val-Thr-	
RV_L b425	--	
V_L425	Met-Thr-Cys	
RV_L a425	Ile-Thr-Cys	
RV_L b425	-----------	

V_L425	Ser-Ala-Ser-Ser-Ser-Val-Thr-Tyr-Met-Tyr	
RV_L a425	Ser-Ala-Ser-Ser-Ser-Val-Thr-Tyr-Met-Tyr	CDR-1
RV_L b425	--	

V_L425	Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Ser-Ser-Pro-	
RV_L a425	Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Lys-Ala-Pro-	
RV_L b425	--	FR-2
V_L425	Arg-Leu-Leu-Ile-Tyr	
RV_L a425	Lys-Leu-Leu-Ile-Tyr	
RV_L b425	-------------------	

V_L425	Asp-Thr-Ser-Asn-Leu-Ala-Ser	
RV_L a425	Asp-Thr-Ser-Asn-Leu-Ala-Ser	CDR-2
RV_L b425	---------------------------	

V_L425	Gly-Val-Pro-Val-Arg-Pne-Ser-Gly-Ser-Gly-	
RV_L a425	Gly-Val-Pro-Ser-Arg-Phe-Ser-Gly-Ser-Gly-	
RV_L b425	--	
V_L425	Ser-Gly-Thr-Ser-Tyr-Ser-Leu-Thr-Ile-Ser-	FR-3
RV_L a425	Ser-Gly-Thr-Asp-Phe-Thr-Phe-Thr-Ile-Ser-	
RV_L b425	--------------------Tyr--------------------	
V_L425	Arg-Met-Glu-Ala-Glu-Asp-Ala-Ala-Thr-Tyr-Tyr-Cys	
RV_L a425	Ser-Leu-Gln-Pro-Glu-Asp-Ile-Ala-Thr-Tyr-Tyr-Cys	
RV_L b425	--	

V_L425	Gln-Gln-Trp-Ser-Ser-His-Ile-Phe-Thr	
RV_La425	Gln-Gln-Trp-Ser-Ser-His-Ile-Phe-Thr	CDR-3
RV_L b425	----------------------------------	

V_L425	Phe-Gly-Ser-Gly-Thr-Lys-Leu-Glu-Ile-Lys	
RV_L a425	Phe-Gly-Gln-Gly-Thr-Lys-Val-Glu-Ile-Lys	FR-4
RV_L b425	--	

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FIG. 4-2
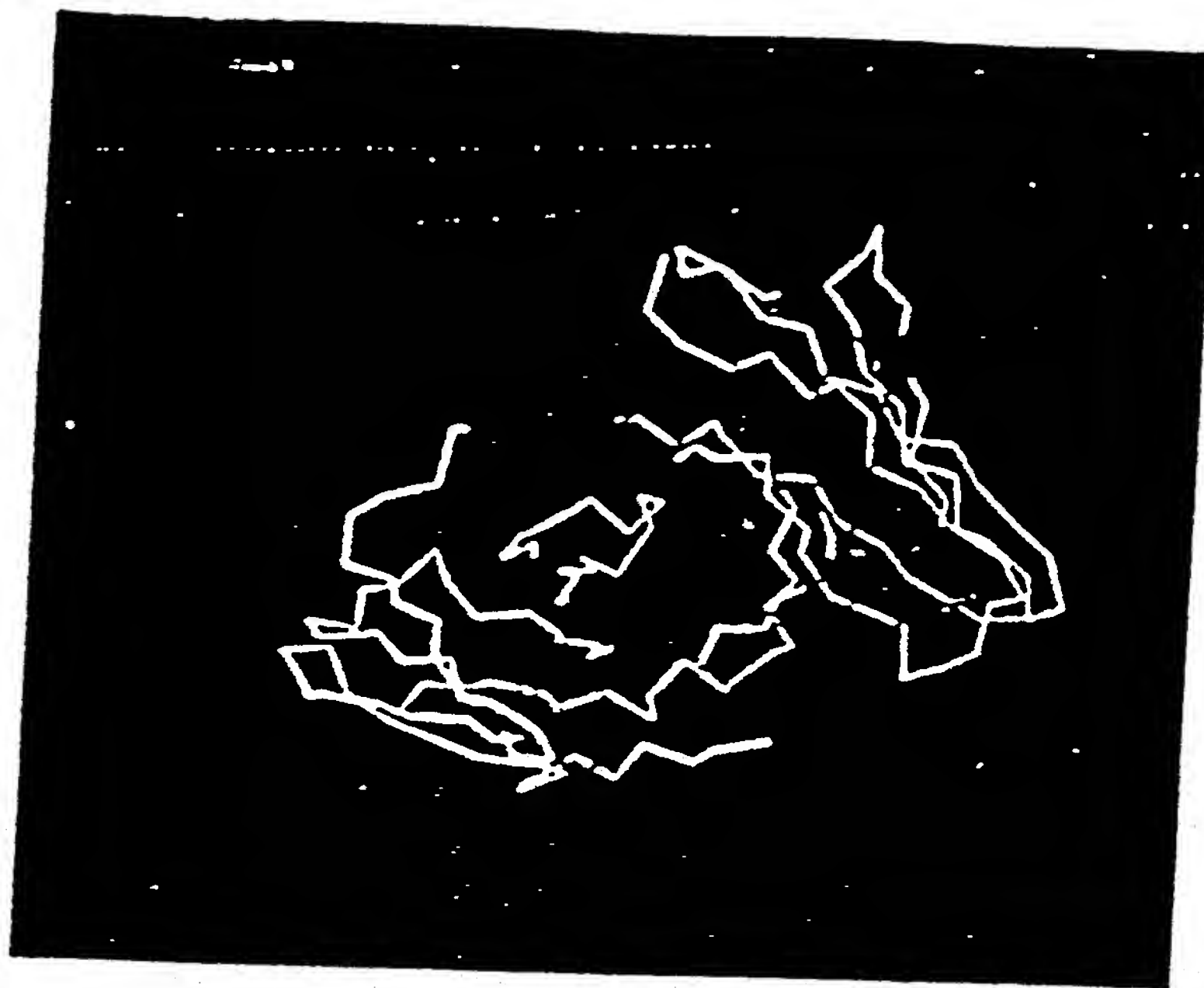
Panel B:

```
Vн 425          Gln-Val-Gln-Leu-Gln-Gln-Pro-Gly-Ala-Glu-
RVн a-d,f 425   Gln-Val-Gln-Leu-Val-Gln-Ser-Gly-Ala-Glu-
RVн e,g-i 425   -------------------------------------------
Vн 425          Leu-Val-Lys-Pro-Gly-Ala-Ser-Val-Lys-Leu-      FR-1
RVн a-d,f 425   Val-Lys-Lys-Pro-Gly-Ala-Ser-Val-Lys-Val-
RVн e,g-i 425   -------------------------------------------
Vн 425          Ser-Cys-Lys-Ala-Ser-Gly-Tyr-Thr-Phe-Thr
RVн a-d,f 425   Ser-Cys-Lys-Ala-Ser-Gly-Tyr-Thr-Phe-Ser
RVн e,g-i 425   ---------------------------------------Thr


Vн 425          Ser-His-Trp-Met-His                           CDR-1
RVн a-i 425     Ser-His-Trp-Met-His


Vн 425          Trp-Val-Lys-Gln-Arg-Ala-Gly-Gln-Gly-Leu-
RVн a-c,h,i 425 Trp-Val-Arg-Gln-Ala-Pro-Gly-Gln-Gly-Leu-
RVн d-g 425     -------------------------------------------
Vн 425          Glu-Trp-Ile-Gly                               FR-2
RVн a-c,h,i 425 Glu-Trp-Val-Gly
RVн d-g 425     --------Ile----


Vн 425          Glu-Phe-Asn-Pro-Ser-Asn-Gly-Arg-Thr-Asn-
RVн a-i 425     Glu-Phe-Asn-Pro-Ser-Asn-Gly-Arg-Thr-Asn-
Vн 425          Tyr-Asn-Glu-Lys-Phe-Lys-Ser
RVн a-i 425     Tyr-Asn-Glu-Lys-Phe-Lys-Ser                   CDR-2


Vн 425          Lys-Ala-Thr-Leu-Thr-Val-Asp-Lys-Ser-Ser-
RVн a,d,e 425   Arg-Val-Thr-Met-Thr-Leu-Asp-Thr-Ser-Thr-
RVн b,h 425     -------------------Val--------------
RVн c,f,g,i 425 Lys-Ala-----------Val---------------
Vн 425          Ser-Thr-Ala-Tyr-Met-Gln-Leu-Ser-Ser-Leu-
RVн a,d,e 425   Asn-Thr-Ala-Tyr-Met-Glu-Leu-Ser-Ser-Leu-     FR-3
RVн a,d,e 425   -------------------------------------------
RVн c,f,g,i 425 -------------------------------------------
Vн 425          Thr-Ser-Glu-Asp-Ser-Ala-Val-Tyr-Tyr-Cys-Ala-Ser
RVн a,d,e 425   Arg-Ser-Glu-Asp-Thr-Ala-Val-Tyr-Tyr-Cys-Ala-Ser
RVн a,d,e 425   --------------------------------------------------
RVн c,f,g,i 425 --------------------------------------------------


Vн 425          Arg-Asp-Tyr-Asp-Tyr-Asp-Gly-Arg-Tyr-Phe-Asp-Tyr  CDR-3
RVн a-i 425     Arg-Asp-Tyr-Asp-Tyr-Asp-Gly-Arg-Tyr-Phe-Asp-Tyr


Vн 425          Trp-Gly-Gln-Gly-Thr-Thr-Leu-Thr-Val-Ser-Ser      FR-4
RVн a-i 425     Trp-Gly-Gln-Gly-Thr-Leu-Val-Thr-Val-Ser-Ser
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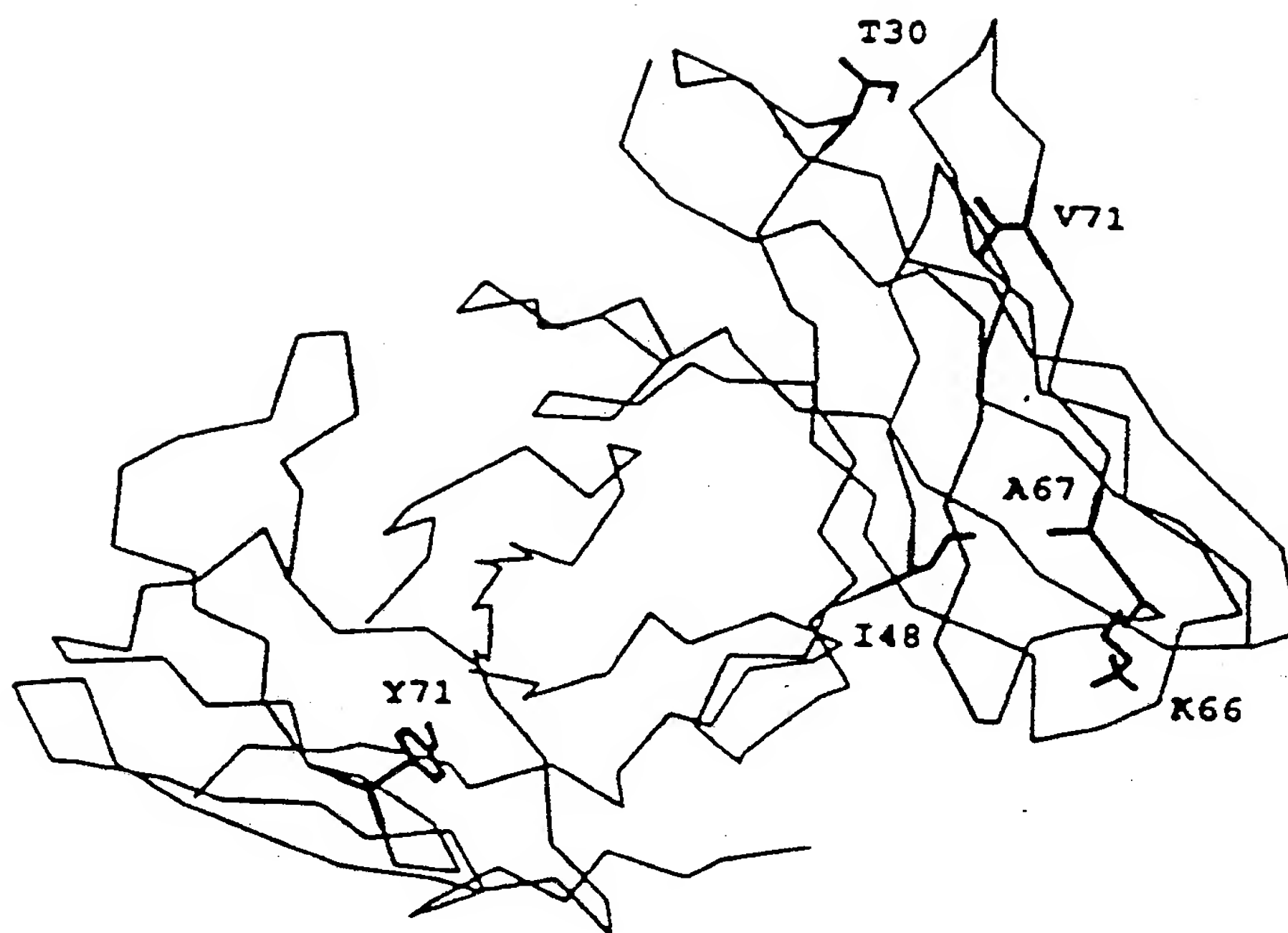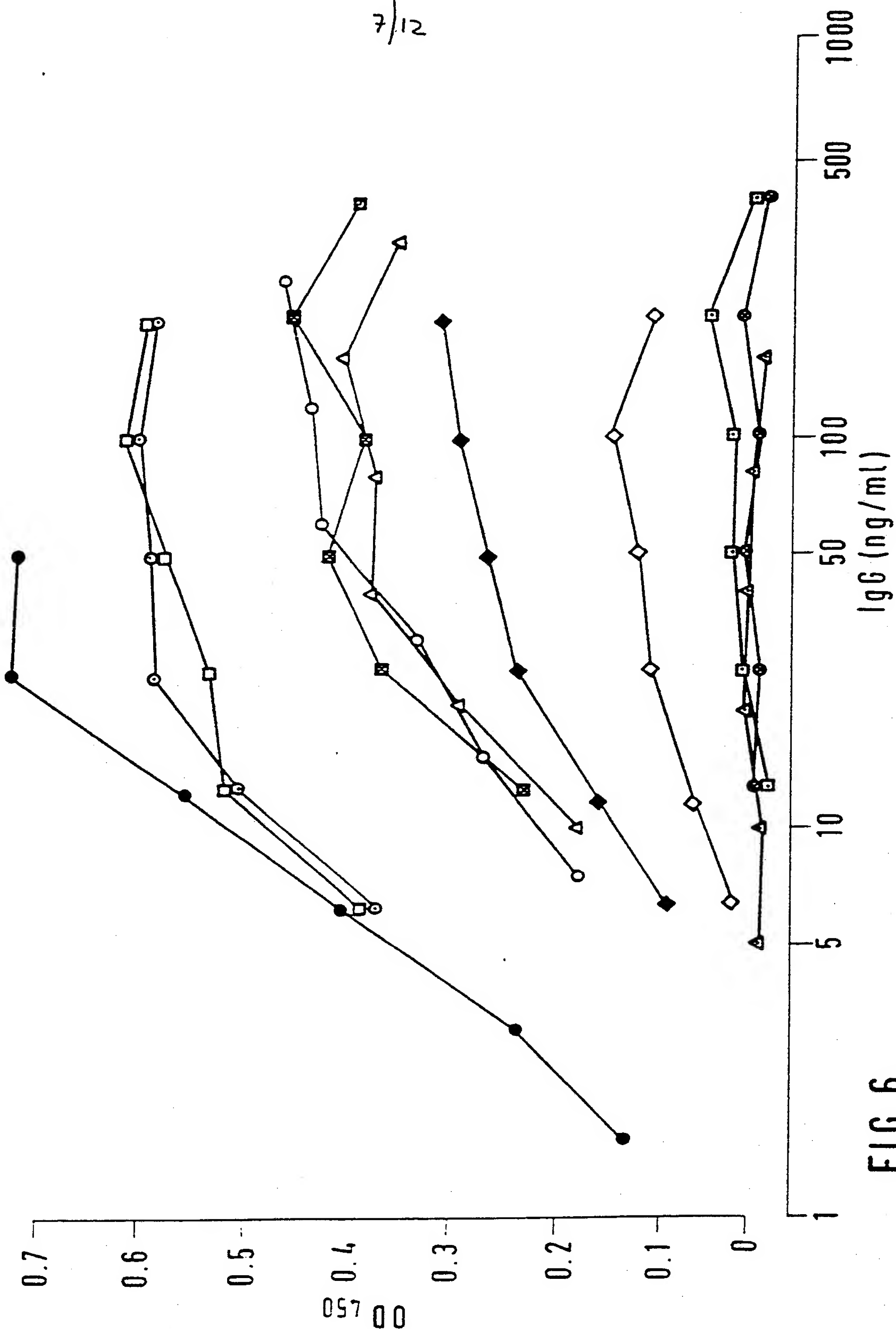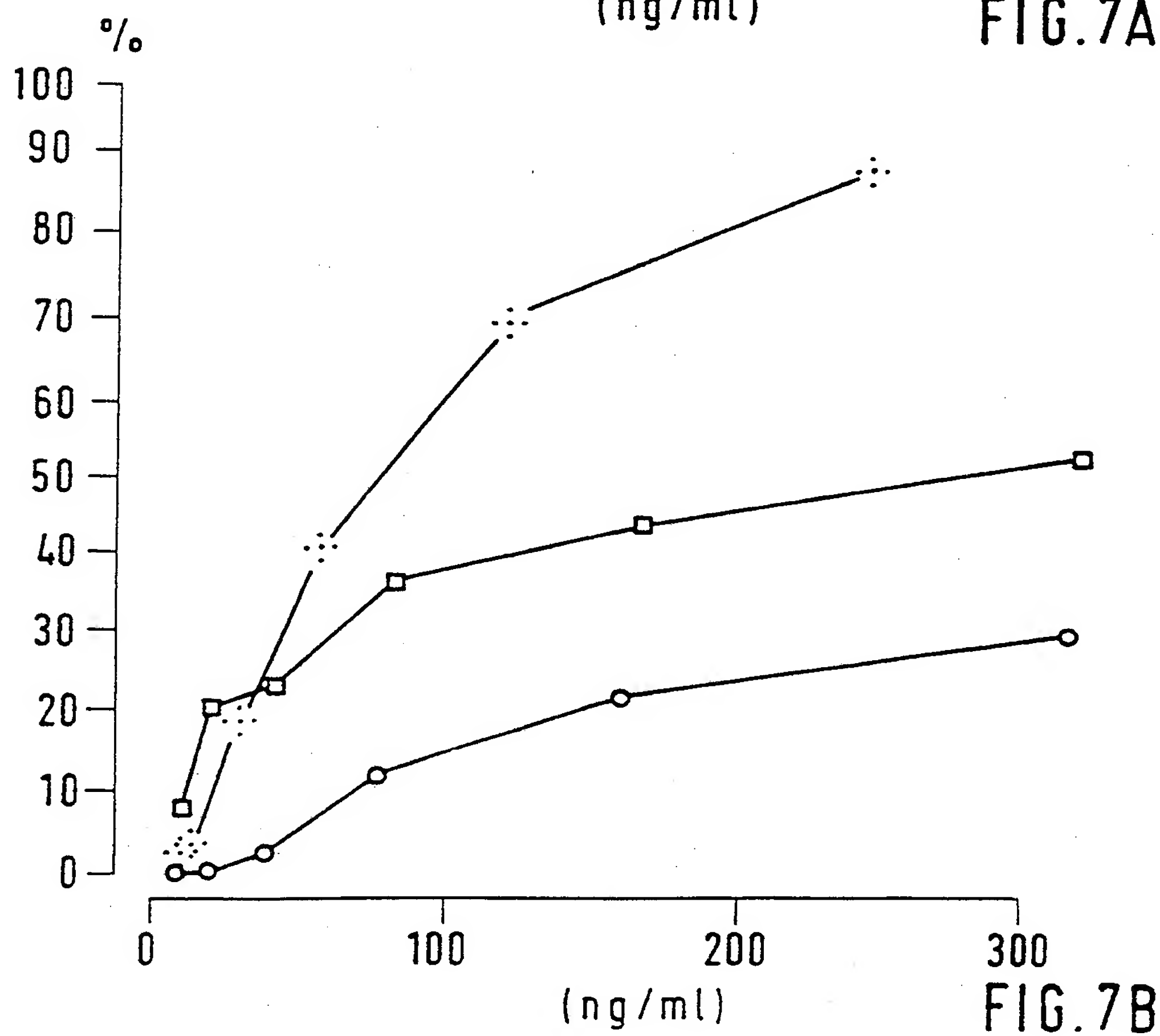
SUBSTITUTE SHEET

FIG. 5

A

B

FIG.6

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FIG.7A

FIG.7B

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FIG.8A

FIG.8B

FIG. 9 A

FIG. 9 B

FIG. 10

FIG.11

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER   (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5	C 12 N 15/13	C 07 K 13/00	C 12 N 15/62
C 12 P 21/08	A 61 K 39/395	A 61 K 49/00	

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document, [11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Journal of Molecular Biology, vol. 215, no. 1, 5 September 1990, A. TRAMONTANO et al.: "Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins", pages 175-182, see paragraph 7: "Conclusion"	1-3
Y	---	4-25
Y	Arch. Biochem. Biophys., vol. 252, no. 2, 1 February 1987, U. MURTHY et al.: "Binding of an antagonistic monoclonal antibody to an intact and fragmented EGF-receptor polypeptide", pages 549-560, see the whole document (cited in the application) --- -/-	4-25

[o] Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
03-06-1992	3 0. 06. 92

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Nicole De Bie

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Science, vol. 239, 25 March 1988, M. VERHOEYEN et al.: "Reshaping human antibodies: grafting an antilysozyme activity", pages 1534-1536, see the whole document, especially figures 1c,2 (cited in the application)	1-3
Y	---	4-25
Y	J. Cell. Biochem., vol. 44, no. 2, October 1990, U. RODECK et al.: "Monoclonal antibody 425 inhibits growth stimulation of carcinoma cells by exogenous EGF and tumor-derived EGF/TGF-alpha", pages 69-79 ---	4-25
X	Proc. Natl. Acad. Sci. USA, vol. 86, December 1989, C. QUEEN et al.: "A humanized antibody that binds to the interleukin 2 receptor", pages 10029-10033, see the whole document (cited in the application)	1-3
Y	---	4-25
P,X	Protein Eng., vol. 4, no. 7, October 1991, C.A. KETTLEBOROUGH et al.: "Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation", see the whole document ---	1-25
A	Nature, vol. 342, 21/28 December 1989, C. CHOTHIA et al.: "Conformations of immunoglobulin hypervariable regions", pages 877-883 (cited in the application) ---	1-25
A	Cancer Res., vol. 47, no. 10, 15 May 1987, A. BASU et al.: "Presence of tumor-associated - antigens in epidermal growth factor receptors from different human carcinomas", pages 2531-2536 -----	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] **OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE** [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claim numbers .... because they relate to subject matter not required to be searched by this
   Authority, namely:

   Although claim 25 is directed to diagnostic methods practised on the human body (Rule 39.1(IV) PCT) the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claim numbers .... because they relate to parts of the International application that do not comply
   with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. [ ] Claim numbers .... because they are dependent claims and are not drafted in accordance with
   the second and third sentences of PCT Rule 6.4(a).

VI. [ ] **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims
   of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this International search report covers only
   those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to
   the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not
   invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) - P9412B 05/91